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Vascular Tissue Engineering in Metabolic Syndrome Conditions – in vitro Studies

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VASCULAR TISSUE ENGINEERING IN METABOLIC SYNDROME CONDITIONS – IN VITRO STUDIES

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ABSTRACT

Cardiovascular disease (CVD) is on the rise in the US and is the leading cause of death worldwide [1]. Associated disorders including stenosis of the vasculature and formation of atherosclerotic plaques can necessitate the need for repair or replacement. Tissue engineering, based on stem cells and scaffolds, is an emerging strategy for the treatment of vascular diseases. Patients who typically require vascular replacement often have comorbidities such as diabetes and hypertension. There remains the challenge of understanding the fate of tissue engineered vascular grafts (TEVGs) in a harsh diabetic environment.

In these experiments we aimed to develop an in-vitro vascular bioreactor model to 1) condition our tissue engineered vascular grafts and 2) model both a hyperglycemic and hypertensive environment. Our goal was to illicit an appropriate response from the vascular grafts caused by exposure to these pathologic environments to better inform us regarding the fate of our grafts before requiring an in-vivo model.

ACKNOWLEDGMENTS

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CHAPTER ONE: INTRODUCTION AND BACKGROUND

1.1 The Impact of Cardiovascular Disease

In the United States, cardiovascular disease (CVD) is on the rise. In 2017, the American Heart Association reported CVD as the underlying cause of 801,000 deaths in the US, in other words, every 1 in 3 deaths [2]. CVD includes stroke, atherosclerosis, congenital heart disease, coronary artery disease and peripheral artery disease. Of specific interest, peripheral artery disease (PAD), refers to partial or complete obstruction of arteries. PAD is usually caused by atherosclerosis and the most common symptom of PAD is intermittent claudication. Patients with diabetes are among those most likely to develop PAD and if left untreated, critical limb ischemia (CLI) can develop and lead to ulceration and in extreme cases amputation [3,4].

1.2 Anatomy and Physiology

The vascular system consists of blood vessels working to carry blood to and from the heart. Within this system, both arteries and veins have three distinct tissue layers; the tunica externa, tunica media, and tunica intima. The tunica intima, the innermost layer, is comprised of the endothelium and the sub endothelial layer [5]. The endothelium, a continuous layer of endothelial cells, lines the vessel and acts as a permeable barrier between the vessel wall and blood, working to regulate the transport of fluid, protein and cells into and out of tissues [6]. Beneath this layer, the sub endothelial layer acts to bind

the endothelium to the connective tissue. The structure of this tunic allows for specific functionality in regulating circulatory function within the vascular system. Moving outwards from the tunica intima, the tunica media surrounds the intima separated only by the internal elastic lamina. This layer is primarily composed of layers of vascular smooth muscle cells. In muscular arteries, as compared to elastic arteries, this layer is typically thicker whereas the media in a vein would typically be thinner and less pronounced. The outermost layer, separated by the external elastic lamina, is comprised of areolar connective tissue with residential fibroblasts as well as vascular smooth muscle cells [7].

1.3 Vascular Disease Pathology

Vascular diseases are characterized by the narrowing or complete obstruction of the vessel lumen. Gradual weakening of the vessel walls can lead to dilation or rupture. A major mechanism contributing to this is arteriosclerosis, literally meaning "hardening of the arteries". Arteriosclerosis has three major patterns; a) arteriolosclerosis referring to the thickening of small arteries and arterioles, b) medial calcific sclerosis, which is typically age related and not involving the lumen and c) atherosclerosis, the development of lipid based fatty plaques [8]. The progression of atherosclerosis, arguably the most important factor, is characterized by a "response-to-injury" hypothesis in which the arterial walls respond to chronic inflammation initiated by damage to the endothelium [9].

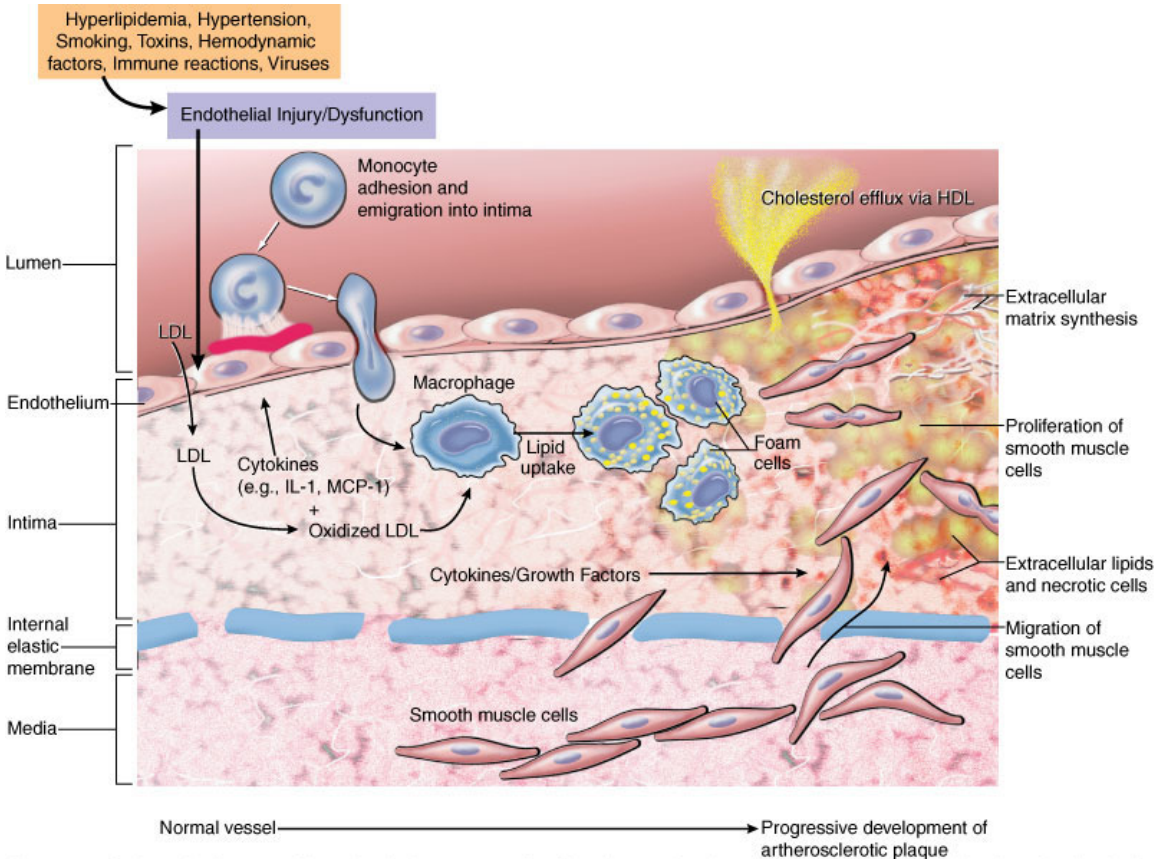

Figure 1: Hypothesized "Response-to-Injury" diagram

Schematic diagram of hypothetical "Response-to-Injury" sequence of events leading to the development of atherosclerosis. [8]

Chronic endothelium injury begins subtly, usually due to associated CVD risk factors. These can include smoking, hypertension, and hyperlipidemia which can lead to altered hemodynamics. This injury marks the beginning of the activation of the endothelium, a phase characterized by increased permeability, leukocyte adhesion, greater thrombotic

potential as well as the adhesion of blood monocytes to the endothelium. Once adhered, the monocytes and other leukocytes begin to migrate into the intima and transform into macrophages and foam cells. Macrophages engulf lipoproteins, specifically the low density lipoproteins (LDL), as well as secrete IL-1 and tumor necrosis factor (TNF) that increases leukocyte adhesion. As the endothelium is increasingly damaged, platelets begin adhering to the endothelium and become activated. Smooth muscle cells respond to the release of factors and activation of the platelets and begin migrating from the media towards the intima. Proliferation of the SMCs leads to increased deposition of extracellular matrix resulting in the formation of an atherosclerotic plaque [8]. At this stage there are key functional consequences; atherosclerotic formation weakens the arterial walls which can lead to a bulging of the walls. Overtime this can result in the formation of an aneurysm which puts the patient at risk of rupture. Clinically silent plaques on the wall, termed "stable", become an issue only when disrupted, thus becoming "vulnerable" [10]. The dislodgement of the plaque can result in ischemia, stroke or myocardial infarction dependent upon the final resting location [11].

1.3.1 Carotid Artery Pathology

The carotid artery is a major artery plagued by atherosclerotic formation and blockage of this artery is a leading cause of stroke and resulting death [12]. The carotid bifurcation, a region where blood flow divides and fluid velocity patterns are more complex, is a common location of atherosclerotic formation, thus suggesting the geometry of the vessel as well as the fluid dynamics plays a key role in inciting formation [13]. In large arteries and areas away from major bifurcations the typical wall shear stress (WSS) experienced

ranges from 20 to 40 dyn/cm^2. However, in regions where flow is disturbed or flow is separated, shear stresses can be zero in the separation region and as high as 50 dyn/cm^2 in the surrounding area. [11]

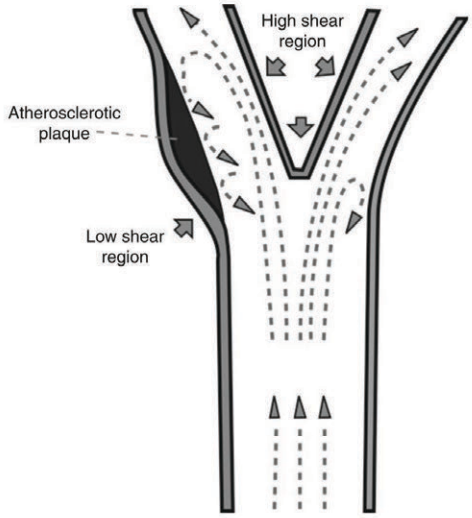

Figure 2: Carotid Artery Bifurcation

Effect of geometry of carotid artery bifurcation on resulting shear stresses within the vessel leading to formation of atherosclerotic plaque [13].

Endothelial cells, as mentioned previously, are highly responsive to changes in blood flow and react accordingly to regulate vasomotion. It is hypothesized that as a result of these low WSS experienced, endothelial cells undergo a change in morphology as well as potentially increase the permeability of the vessel leading to a region more susceptible to atherosclerotic formation [13].

1.4 Impact of Diabetes in Cardiovascular Disease

Diabetes Mellitus (DM), characterized by insulin resistance or lack thereof is a huge contributor to the progression of vascular disease. There are three main classes of DM: Type 1, Type 2 and gestational. Type 1 Diabetes results from insufficient production of insulin due to an autoimmune response that destroys the pancreatic beta cells. Type 2 Diabetes, the more common form is characterized by a resistance to insulin and accounts for 90-95% of cases worldwide [14]. Patients with DM are ten times more at risk for developing a CVD in their lifetime. A key factor contributing to this is the diabetes-associated accelerated atherosclerosis. Although the underlying mechanisms are not completely understood, there are many key contributing factors that play a role in the progression of atherosclerosis and CVD as shown in the figure below [15].

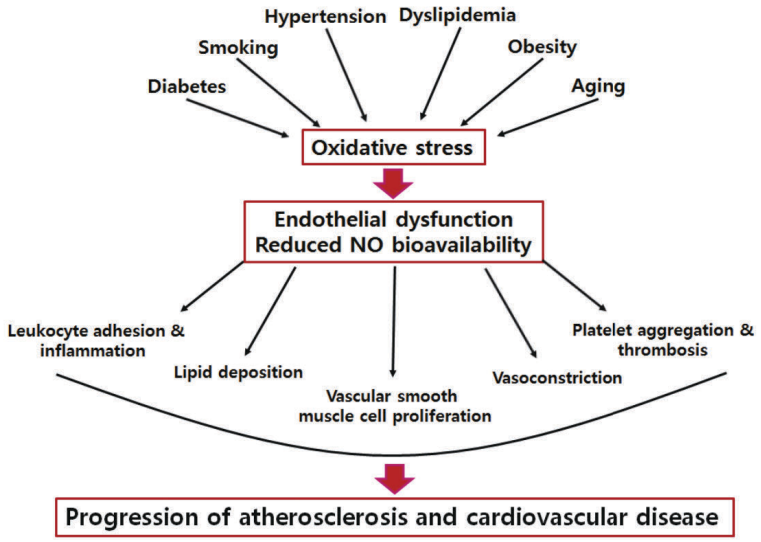

Figure 3: Progression of Atherosclerosis and Cardiovascular Disease

Overview of progression of risk factors leading to atherosclerosis and cardiovascular disease [15].

1.4.1 Endothelial Dysfunction

Endothelial dysfunction refers to the activation of endothelial cells, representing a switch from a quiescent phenotype to an activated defense response from the host [16]. Most cardiovascular risk factors are associated with an increase in reactive oxygen species (ROS) which leads to oxidative stress of the endothelium [15]. Production of ROS such as H_2O_2 and prolonged exposure to them can induce senescence of the ECs leading to loss of integrity as well as detachment. Increase of ROS is also responsible for aggravating inflammation of the vasculature, which further promotes leukocyte adherence and migration. Monocytes are also increasingly recruited and begin to switch phenotypes to transform into foam cells. These issues lead to greater damage to the endothelium and provide a basis for developing atherosclerosis and other cardiovascular diseases as shown in **Fig.3** [15].

1.4.2 Hyperglycemia

Type 2 diabetes is characterized by insufficient production of insulin to counter target organ insulin resistance. Inability of the body to maintain appropriate glucose levels leads to elevated blood glucose levels referred to as hyperglycemia [17]. Vascular injury and increased inflammation are devastating responses to the increase in glucose levels. Hyperglycemia enhances the expression of inflammatory genes to promote the attraction and adhesion of monocytes seen in the early stages of atherosclerosis [14]. It is the dysfunction at a cellular level that is believed to elicit such a detrimental response. An inevitable trait of hyperglycemia is the production of advanced glycation end products (AGEs). AGE production is normal as aging occurs, however the accumulation of AGEs

is rapidly accelerated in diabetes [18]. High glucose levels lead to increased production of reactive oxidation species (ROS). The typical response to the presence of ROS is the production of antioxidant enzymes but in diabetic conditions this is not always the case [19]. This response coupled together with the imbalance of ROS production results in oxidative stress [19]. It is thought that this oxidative stress in the endoplasmic reticulum leads to an impairment of b-cells function and increased ROS formation [17].

1.4.3 Hypertension

Hypertension is highly prevalent worldwide and common in patients with diabetes. The incidence of hypertension increases in populations with cardiovascular co-morbidities such as diabetes mellitus, and peripheral artery disease [20]. Hypertension is highly correlated with obesity in patients and is linked through mechanisms such as metabolic and vascular dysfunction [21]. Of major note is the role vascular remodeling plays in increased arterial stiffening. This remodeling is characterized by a change in the normal structure of blood vessels to accommodate physical or chemical changes in the environment. Arterial stiffening, although typically associated with increasing age, is found in hypertension as remodeling allows the vessels to withstand higher pressures [22]. This stiffening, caused by the changes in extracellular matrix is well known to increase total peripheral resistance [23]. The major components in the arterial wall are collagen and elastin. Elastin, the dominant protein, allows elastic recoil of the arteries while collagen provides strength to the arterial wall [24]. Elastin is primarily produced during development. As elastin fibers are degraded over time or through tissue damage, production of collagen, a much stiffer protein, is increased. Collagen is 100-1000 times

stiffer than elastin and takes on a greater mechanical load as the elastin degrades [23]. Calcification of the elastic lamellae contributes to increased stiffening. [XU] Current treatment options for hypertension includes prevention of CVD by strict control of blood glucose levels, weight reduction, limiting alcohol intake and to reduce smoking [19]. Recommended target blood pressures for diabetic patients are below 130/80 mmHG [19].

1.5 Management of Diabetes

Approximately 20 million people are affected by diabetes mellitus (DM) in the U.S. [25]. There currently remains no cure for diabetes. Instead, a strict management and regulation of blood glucose level has been shown to be an effective method in reducing microvascular disease, however it has not been shown to reduce myocardial infarction or vascular disease on a macro level [26,27]. Self-monitoring of glucose with the use of blood glucose pumps along with proper insulin administration is essential to an effective treatment [26]. For patients with type 1 DM, treatment includes insulin injections combined with an increase in exercise and diet control. Initial therapy for patients with type 2 DM includes reduction of weight and an increase in physical activity [26].

1.6 Current Engineering Solutions

As diabetes- induced atherosclerotic and calcification leads to greater obstruction, the risk of critical limb ischemia (CLI) increases [28]. This disease, if left untreated, can ultimately lead to limb loss. It is estimated that CLI leads to 35% of major amputations yearly and diabetic patients are 10 times more likely to undergo an amputation [29].

Typically surgical bypass grafting and endovascular treatments are employed as a first approach to reduce the need for amputation [30]. Vascular stents are metal frameworks that act to support the lumen of the vasculature from within. Bare metal stents (BMS) are typically comprised of nitinol, a material that self-expands at physiological temperatures. [add reference for nitinol]. Combined with balloon angioplasty, stents act to prevent restenosis from occurring. However, stenting inflicts trauma to the lumen of the vessels which can lead to thrombosis and intimal hyperplasia leading to restenosis of the stent. As an improvement, drug eluting stents (DES) were designed to further prevent restenosis by eluting drugs such as Sirolimus and Paclitaxel, which have been shown to improve the outcome of coronary procedures. However, the anti-proliferation properties of these drugs lead to decreased healing of the endothelium after trauma. [add reference]

Advances in the last decade have shifted the open surgery grafting approach to a percutaneous endovascular treatment associated with lower morbidity. The continuous development and improvement of vascular stents has greatly improved the outcome of these percutaneous procedures [29]. However, in recent studies it has been shown that for a diabetic population, coronary artery bypass grafting (CABG) is the preferred revascularization procedure [26]. Due to the systemic effect of the vascular disease, many patients do not have viable homologous donor arteries. In these cases, synthetic materials are turned to as alternative. There are many approaches to provide treatment for the degradation of the vasculature. Some of these treatments include the use of autologous grafts and synthetic grafts as replacements. Although these practices are commonly used, they do have their limitations and complications. Current synthetic graft approaches

include the use of polytetrafluoroethylene (PTFE) based vascular grafts such as Gore-Tex^{TM} as well as using Dacron and other polymeric materials. These approaches are limited by their inability to grow with the patient as well as limitation due to higher levels of thrombotic complications in the smaller diameter models [31,32].

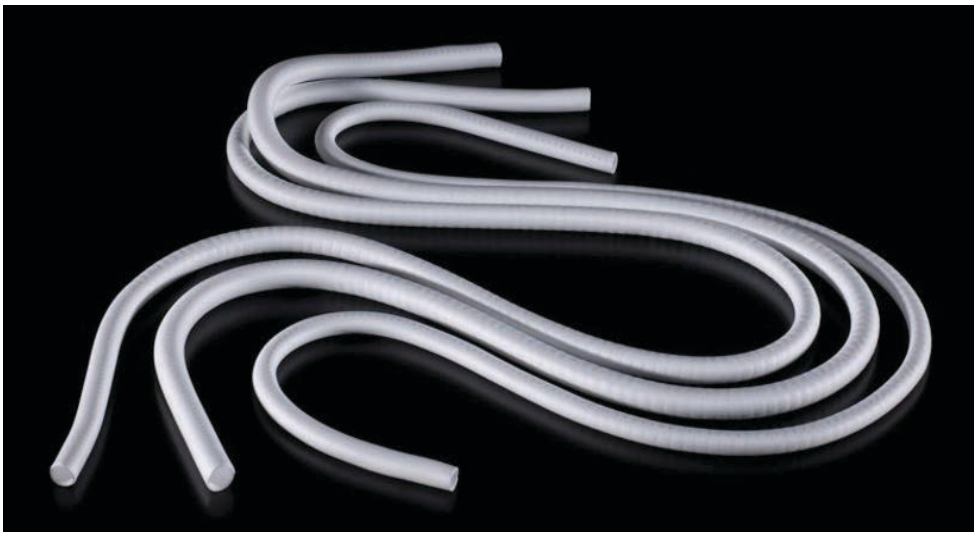

Figure 4: Alternative Vascular Graft

Current synthetic vascular graft on the market comprised of ePTFE (Gore® Propaten® Vascular Graft)

CHAPTER TWO: VASCULAR TISSUE ENGINEERING

2.1 Vascular Tissue Engineering

Tissue engineering is a rapidly emerging field in vascular therapies. The inadequacy of the aforementioned treatments, the large rate of revision surgeries as well as a lack of autologous vessel sources in a large proportion of the patients has spurred the search for a solution [33]. There is a growing need for a functional replacement capable of regeneration. To be considered a functional tissue engineered vascular graft (TEVG), the graft should be non-thrombogenic, non-immunogenic and exhibit good mechanical strength as well as vasoreactivity. The specific tri-layer design should be maintained ideally down to the cellular level with residential fibroblasts and smooth muscle cells as well as a continuous endothelial cell lining the intima [33,34].

2.1.1 Paradigm

The general scaffold-based top down approach begins first with the isolation of a suitable cell source from the patient. These cells are seeded onto a biodegradable scaffold. Following this, the new graft will be conditioned in vitro before implanting back into the original patient as depicted in the figure below [34].

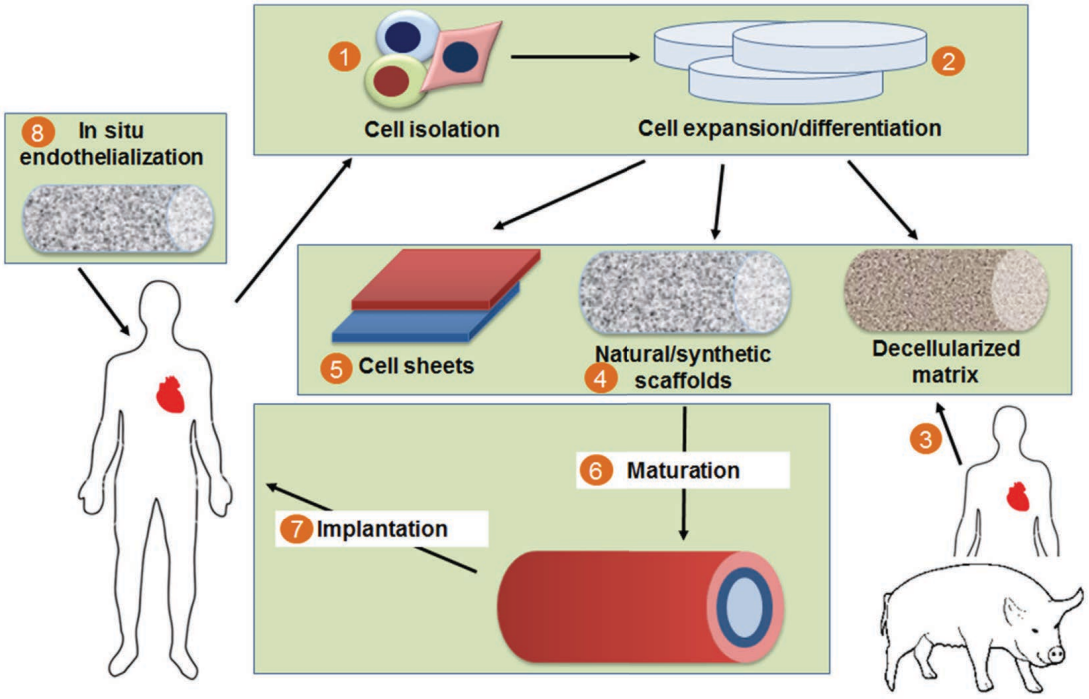

Figure 5: Vascular Tissue Engineering

Paradigm of general approach to producing a patient-tailored vascular graft [34].

2.2 Biologic Scaffolds

The biomaterials used in cardiovascular tissue engineering should be biocompatible and of adequate mechanical strength to withstand stresses [34]. Vascular grafts based on natural polymers, primarily elastin, collagen and fibronectin, are typically biocompatible and are better suited for optimal cell attachment and signaling as compared to synthetic materials [33,34]. Decellularized tissues are of particular interest because of their wide availability, ECM-composition and mechanical strength-retaining structure. Tissues may

be derived from allogeneic or xenogeneic sources and decellularization achieved by washing the tissues with detergents and buffers [33]. Because these scaffolds are derived from natural materials, degradation of the tissue does not result in harmful products. In fact, the rapid degradation and ability for replacement is an important attribute of ECM-based scaffold leading to constructive remodeling of the tissues [35].

2.3 Cell Populations and Seeding

There are many options available for a suitable cell population to revitalize the chosen scaffold. An ideal source needs to be non-immunogenic, easily harvestable, of proliferative nature and possess the ability to differentiate into specified cells [36]. Cells may be allogeneic, xenogeneic or of an autologous source. Autologous cells, the preferable choice, eliminate the risk for immunorejection. However, they may be limited in availability, costly to expand, or have minimal proliferative abilities. Popular sources for stem cells include bone-marrow-derived stem cells, endothelial progenitor stem cells, cardiac stem cells and adipose-derived. Adipose tissue is an ideal source for harvesting autologous stem cells. The harvesting from common adipose deposits results in minimal pain to the patient and yields clinically significant amounts of cells [37]. These cells have been shown to possess high proliferative properties and the ability to differentiate into many vascular cell lineages such as endothelial cells, smooth muscle cells and fibroblasts. Miranville et al. 2004 demonstrated the ability of Adipose-derived stem cells (ASCs) to differentiate into endothelial cells with the addition of growth factors into the culture media growth media. Vascular endothelial growth factor (VEGF) (0.5 ng/ml) and insulin-

like growth factor (IGF) (20ng/ml) promoted the appearance of markers vWF and CD-31 [38]. Wang et al. 2010 induced differentiation of ASCs with administration of transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-4 (BMP4) expression of SMC-related markers α-*SMA* and *SM* myosin heavy chain were observed [39]. ASCs high plasticity makes them highly desirable for repopulating a vascular graft. With the aid of mechanical and chemical cues, there is hope for remodeling and regeneration. With an appropriate cell source, there still remains the challenge of repopulating the scaffold with the cells as well as ensuring adequate protection from subsequent pressures and flows following implantation. Fibrin hydrogels have been investigated as a delivery vessel for cells. Because fibrinogen and thrombin, products of the coagulation cascade, polymerize to form the fibrin gel, this method is promising as the fibrin gel would be biocompatible. Hydrogels have been used to encapsulate cells and provide protection during seeding [40]. The fibrin gel acts to provide efficient application and even cell distribution.

2.4 In-vitro Bioreactor Graft Conditioning

Continuing with the general approach of tissue engineering, after combining the cell source with the scaffold, a bioreactor is a necessary step, owing to the limitations of static culture. At the time of implantation, the graft should be mechanically strong, exhibit vasoreactivity and non-thrombogenecity [33]. Vessel reactors are specifically designed to mature and condition the constructs by mimicking the physiological conditions and stimuli the native vessel would experience.

2.4.1 Mechanical and Biochemical Cues

Bioreactors subject grafts to forces such as pressure, cyclic strain and wall shear stresses to provide necessary cues. The constant cyclic strain induces a more mechanically stable scaffold. This strain is important for conditioning and development of smooth muscle cells within the constructs. Wall shear stress plays a large role in the organization of endothelial cells, as well as their alignment to the direction of flow and maturation on the graft [33,41,42]. As the scaffold is stretched with the pulsatile strain, production of collagen and elastin is stimulated leading to better remodeling of the decellularized vessels [43]. Along with mechanical cues, growth factors may be added to the culture medium of the bioreactor to further encourage differentiation of the seeded cells. In combination with mechanotransduction, these cues are instrumental in allowing the stem cells time to adjust and adapt in the dynamic environment while promoting construct remodeling.

2.4.2 Current TEVG Approaches

Current studies have shown the use of decellularized tissue reseeded with cells to be a promising approach. Tillman et al. 2012 showed positive results in an ovine model. Decellularized porcine carotid arteries were seeded with endothelial progenitor cells followed by preconditioning in a bioreactor for 2 weeks. This was performed prior to implantation to adjust grafts to arterial flows [44]. The importance of in-vitro pre-conditioning was also reported by Kaushal et al. 2010 A gradual increase of shear stress (1 dyn/cm^2 – 25 dyn/cm^2) over 2 days optimized cell retention. Pre-conditioned grafts

were implanted into an ovine model to replace a portion of the carotid artery and all EPC-seeded grafts remained patent up to 130 days after implantation [45]. Many groups recognize the importance of pre-conditioning the tissue-engineered grafts but also understand there are associated drawbacks. The production and operation of bioreactors can be difficult and require extensive training. There can be many components requiring sterilization and multiple steps during the process. Schulte et al. 2014 aimed to build an all-in-one system that allowed simultaneous seeding, rotation and perfusion of the grafts [46]. The all in one design is shown in **Fig.6.** The design fits on a secure base plate that can fit into a cell culture incubator. The seeding chamber is capable of rotation for even cell distribution and can be connected to the main flow after seeding is achieved. The design includes a pump, potentiometer and pulse generator.

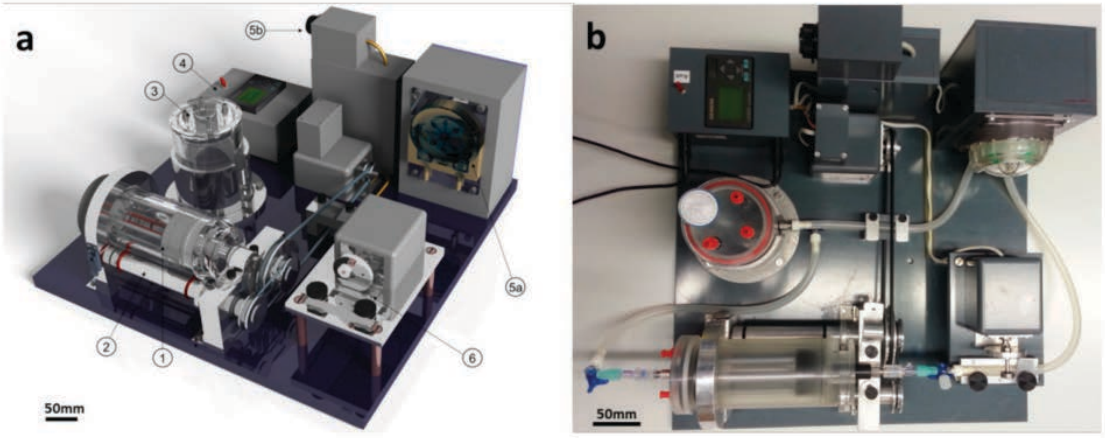

Figure 6: All-In-One Vascular Bioreactor

All-in-one bioreactor build includes 1) seeding device, 2) rotating mixer, 3) media reservoir 4) control unit, 5a) roller pump, 5b) potentiometer, 6) pulse generator.

The combination of tissue engineered grafts and bioreactor systems presents many promising solutions for testing not only grafts, but also medical devices and substances. An ultimate goal for systems like this is to reduce the animal model burden by performing in-vitro testing in specialized systems.

CHAPTER THREE: DEVELOPMENT OF BLOOD VESSEL TISSUE ENGINEERING IN VITRO MODEL

3.1 Rationale

A vascular bioreactor system is necessary to dynamically condition TEVGs as well as to recreate physiologically relevant environments. This chapter explores the development of a vascular bioreactor to simulate a hyperglycemic environment as well as a hypertensive environment.

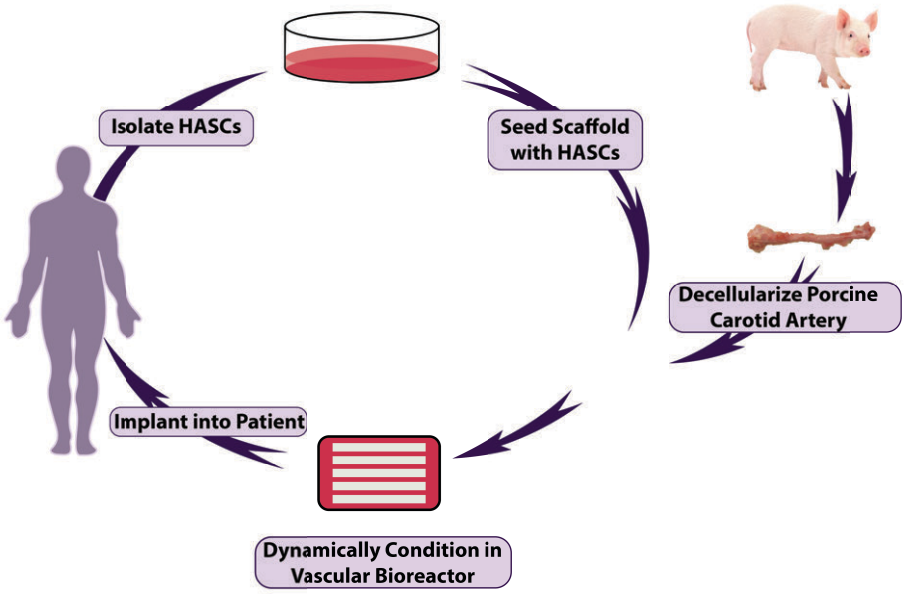

Figure 7: Vascular Tissue Engineering Paradigm

Overview of general approach to producing a patient-tailored vascular graft. Beginning with harvesting of carotid artery from a porcine model and subsequent decellularization. Autologous cells are isolated and expanded before reseeding the vascular graft. The graft is dynamically conditioned in a vascular bioreactor prior to implantation into patient.

3.2 Vascular Bioreactor for Hyperglycemic Conditioning

To mimic physiological conditions in vitro, a vascular bioreactor was previously designed by lab members Dr. James Chow and Dr. Lee Sierad. The bioreactor was designed in SolidWorks and machined by the Clemson Machining and Technical Services.

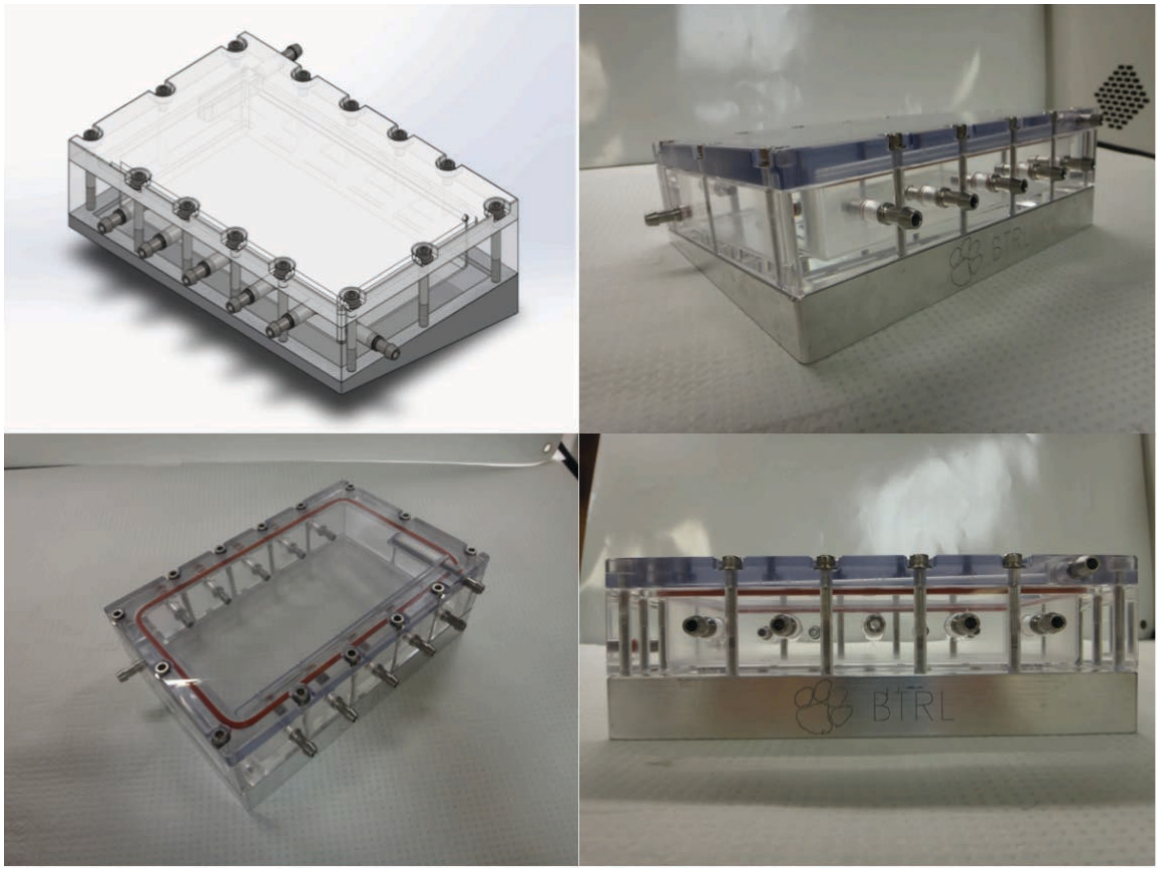

Figure 8: Vascular Bioreactor

Vascular bioreactor developed by James Chow and Lee Sierad. Schematic of design shown in SolidWorks and resulting build.

This newly designed iteration of the main chamber can accommodate 5 vascular grafts up to 4 inches long. Stainless steel "quick" barbed fittings provided a quick through wall connector featuring an o-ring seal for superior sterility. An o-ring at the interface of the body and the lid also provides a tight seal and increased sterility. A sloped aluminum base combined with an air bubble channel grooved into the polycarbonate lid provides ease when filling the chamber. The acrylic body of the chamber can be sterilized with ethylene oxide gas and provides excellent visualization of the grafts. This bioreactor was designed to be constructed quickly while minimizing the risk of contamination. To accommodate a wider range of graft sizes, a minimal adjustment was made to the previous designs. The stainless steel through-wall fittings were outfitted with connections to Leur fittings on one end. This allows for commercially available male Leurs fittings of varying inner diameters to connect to the stainless steel fittings. These fittings can be matched to the vessel grafts prior to mounting in the bioreactor to ensure appropriate fit.

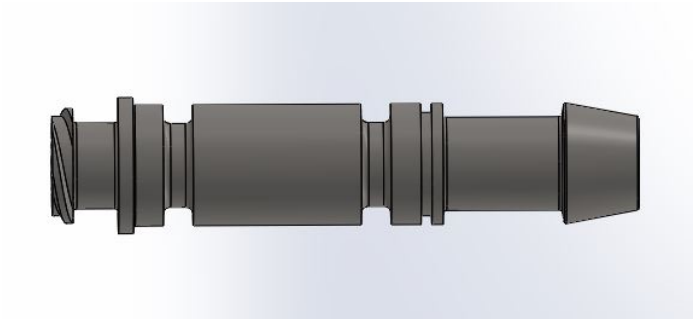

Figure 9: SolidWorks Modification of Through-Wall Fitting

New design rendered in SolidWorks for through-wall fittings to allow quick assembly and interchangeable Leur fittings for better matching of inner diameters.

The new chamber is connected in a flow loop and flow can be controlled through the use of a peristaltic pump. As shown in **Fig.10**, a closed reservoir directly following the pump acts as a pulse dampener to lessen the peristaltic action from the pump. Culture media is perfused across the graft byway of a manifold to divert the flow. The media re-enters and fills the large chamber to completely bathe the grafts. Media exits the chambers and

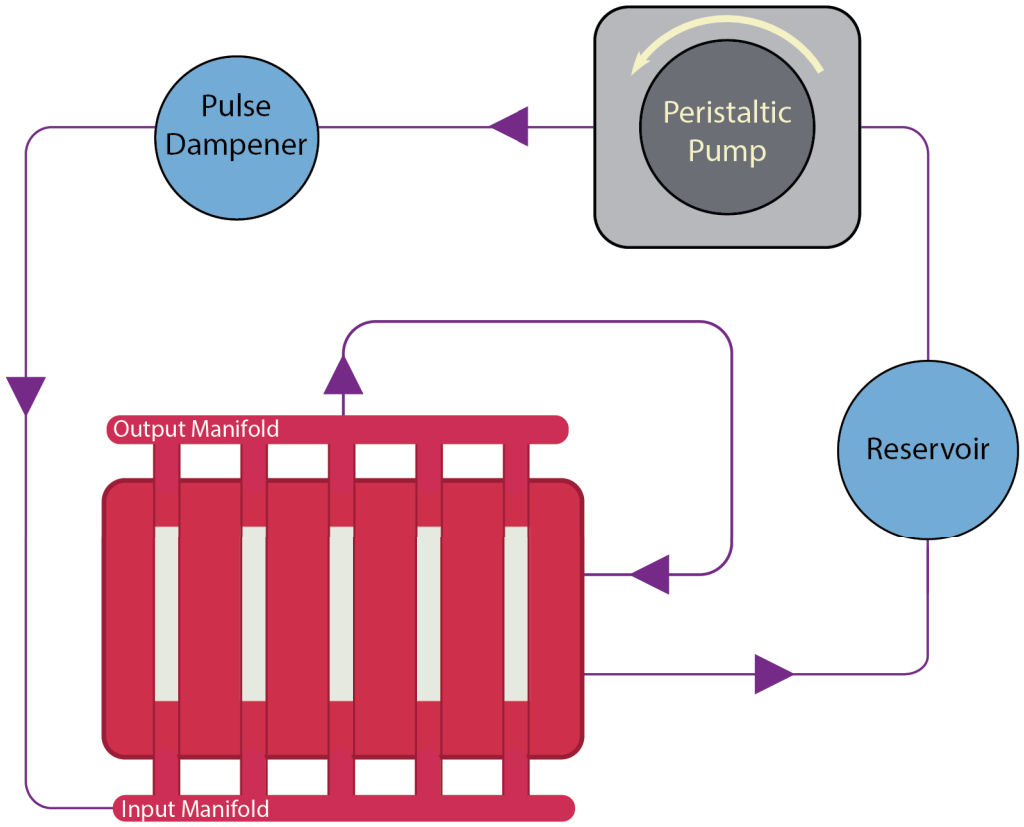

Figure 10: Hyperglycemic Vascular Bioreactor Schematic

General schematic outlining the flow loop and components of the vascular bioreactor used in the hyperglycemic experiments.

enters a reservoir where sterile gas transport can take place via a 0.2μm filter cap atop the reservoir. The media continues back to the peristaltic pump completing the loop.

3.2.1 Advantages and Drawbacks

The major advantage of this design as compared to previous iterations is the number of samples that can be housed in a single reservoir. With one bioreactor system, 5 samples can be run at the same conditions. The main reservoir simplifies assembly much more than if the vessels were housed individually. The compact size allows for easy removal and transportation during media changes. As well, due to its small size, the system easily fits within a standard cell culture incubator. However, this design has its drawbacks. This system requires the use of a peristaltic pump. Traditionally, a large multi-head peristaltic pump (Cole Parmer) is used. Multiple systems can be attached to the peristaltic pump which is convenient to conserve space, however if your conditions require variable flow speeds it will require a separate pump and additional space in an incubator. As well this design is controlled only through the use of the pump's internal settings. For experiments focused on biochemical cues this does not pose much of an issue, but for increased experimentation with biomechanical cues, more sophisticated parameters and controls are needed.

3.3 Vascular Bioreactor for Hypertensive Conditioning

A partnership was enacted with Aptus Bioreactors, a local bioreactor-development company, to improve the vascular bioreactor. Developing design inputs and

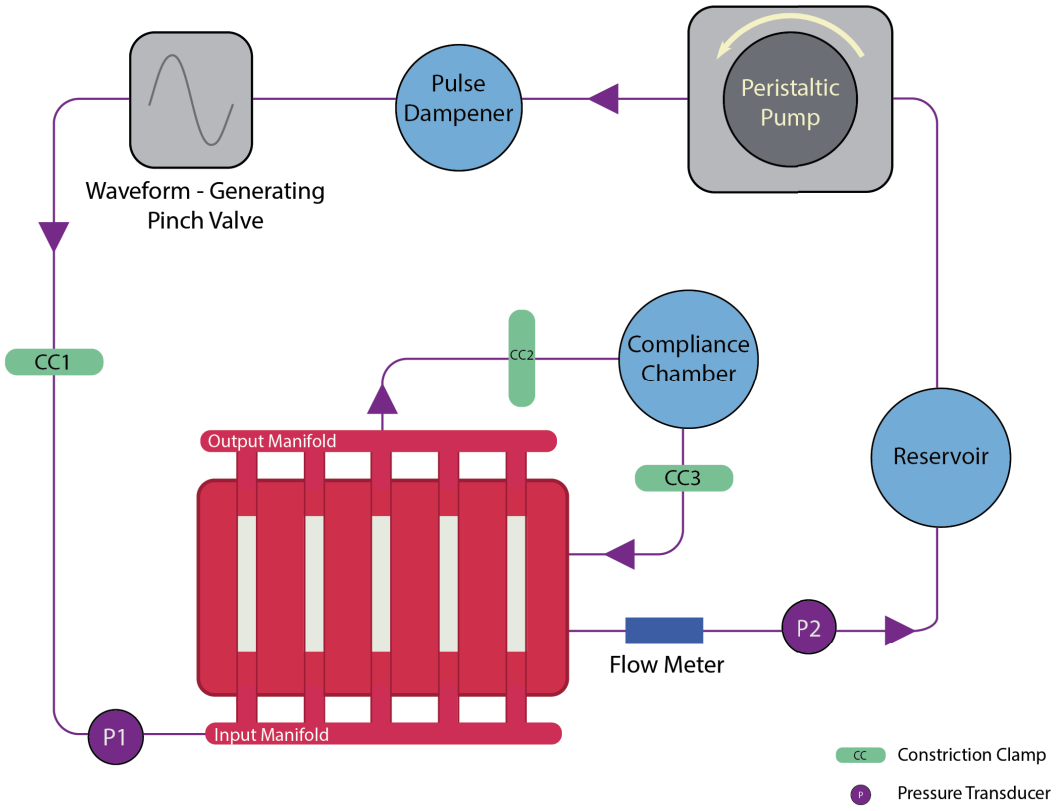

Figure 11: Hypertensive Vascular Bioreactor Schematic

General schematic outlining the flow loop and components of the vascular bioreactor used in the hypertensive experiments.

validating various components or assembled portions of the system were my main objectives during a 3-month internship in the engineering department.

Final manufacturing and assembly was coordinated by Aptus Bioreactors and systems were leased for the duration of the experiments. The previous hyperglycemic vascular bioreactor was outfitted with new controls to have a wider control over the parameters. This new iteration would allow us to experiment with various pressures and flows to better model physiological conditions.

3.3.1 Bioreactor Additions and Development

As compared to the previous iteration, the hypertensive bioreactor needed additional capabilities. The pressure waveform is a complex parameter that can be affected by minute changes in constriction and even tubing compliance [47]. By modifying the location and magnitude of constriction, diastolic and systolic pressures can be modulated. Peristaltic pumps allow the isolation of the circulating fluid from the pump by the action of roller heads directly on the tubing of the flow loop. Addition of a pulse dampener following the peristaltic pump acts to reduce the noise introduced by the pump as well as to smooth out high and low frequency peaks. The reduction of noise depends on the presence and volume of air within the pulse dampener. Glass vessels were designed with removable caps to allow adjustment of air levels to optimize the noise reduction as shown in **Fig.12**. Additional compliance chambers were added to aid in the modulation of pressures. Three constriction clamps distributed in the system as shown in **Fig.11** act as the primary control

A pinch valve modifies the amplitude of the pressure wave also known as the pulse pressure. As seen in **Fig.11**, these components are now included in the design. However, the addition of these components resulted in the need for a system to control and collect the data. Using Aptus Vascular Software we could simultaneously monitor flow rate, two pressures and modulate the pinch valve.

Figure 12: Glass Pulse Dampener

Pulse dampener constructed of glass with removable cap to adjust fluid levels and inlet and outlet to connect to flow loop.

3.3.2 Fittings Modifications

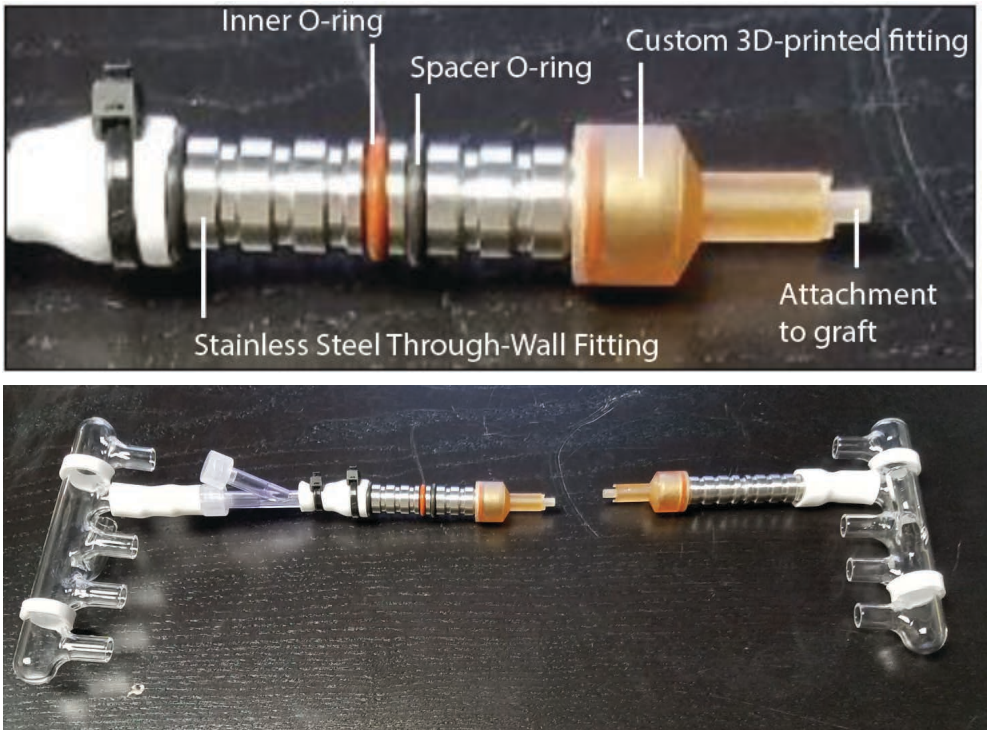

Figure 13: SolidWorks Modification of Through-Wall Fitting

New through-wall fittings designed by Aptus Biroeactors coupled with attached custom 3D-printed fitting. O-rings shown to indicate appropriate operation.

An upgraded design of the through-wall fittings was implemented. With the increase of flow and pressure required by our new final parameters, it is important that the flow from manifold to fitting and through the vessel is as seamless as possible. New stainless steel fittings were designed by Aptus for a more seamless flow and are used combination with the new custom 3D-printed fittings to transition from fitting to the vessel. The new fittings can be matched to the diameter of the graft's lumen, and unlike traditional Leur

style fittings, the new fittings do not have a large constriction of flow. This new design allows for a more relevant modeling of flow through the vascular grafts.

3.3.3 Advantages and Drawbacks

This new iteration of the bioreactor has a few key major improvements. With the addition of the constriction clamps, and pinch valve, pressures and flows can be adjusted and monitored. The ability to monitor and adjust these parameters provides more relevant physiological conditions than the previous iterations. As well, this design is much more compact due to the fact that the controlling unit contains the electronic system, all ports for cables as well as a peristaltic pump. The included pump is much smaller than previously used pumps which allows for an individual pump to be used in each system and to still easily fit within a cell culture incubator. There are still some modifications that can be done to further improve these systems. The constriction clamps are currently manually controlled. These must be loosened during media changes and repositioned after. Eventually, electrically controlled constriction clamps would be preferable as they could modulate the constriction to modify the pressures automatically.

CHAPTER FOUR: HYPERGLYCEMIC BIOREACTOR

4.1 Rationale and Hypothesis

Tissue engineered blood vessels are exposed to harsh environments upon implantation in diabetic patients. Our aim is to simulate the modification of the TEVG that would occur in this pathological environment. We hypothesize that the high glucose environment simulated in our in-vitro model will alter the efficacy of differentiation of adipose stem cells into targeted vascular cells.

4.2 Materials and Methods

4.2.1 Vascular Graft Preparation

Fresh porcine carotid arteries obtained from Animal Technologies, Inc. (Tyler, TX), Excess fat was removed from the arteries followed by incubation in 0.1M NaOH solution at 37°C for 24 hours. This was followed by extensive rinsing with deionized water until the pH of the rinse water dropped to neutral. Scaffolds were stored in sterile 1X PBS. This treatment removed cellular epitopes and most of the collagen, leaving vascular elastin fibers unaltered. Half of the arterial elastin scaffolds were treated with sterile 0.1% PGG in 50nM 4-(2-hydrozyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 5.5, containing 20% isopropanol, overnight at room temperature under agitation and protected from light. Afterwards, scaffolds were rinsed in sterile 1X PBS. They were then stored in sterile 1X PBS containing 1% protease inhibitors and 1% Penicillin-Streptomycin at 4°C.

4.2.2 In-vitro Proliferation of HASCs

Normal culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (glucose content 1g/L) with 10% fetal bovine serum and 1% Penicillin-Streptomycin. No additional glucose was supplemented. Diabetic media was generated by adding D-(+)-Glucose Monohydrate to a concentration of 5.5 g/L. ASCs were plated at a density of 5000 cells/cm^2 and cultured in either control or diabetic media for up to 4 weeks on tissue culture plastic.

4.2.3 Vascular Graft Seeding

Ten vascular grafts (~6- 7cm in length) were prepared for seeding. Barbed Leur fittings of varying sizes were matched to the ends of each graft and zip tied to secure the graft. The decellularized elastin scaffolds were seeded to both the adventitia and the lumen with expanded hASCs to yield a vascular graft. A total of ($3x10^4/mm^2$) ASCs were injected into the media at varying sites along the scaffold with a 30-gauge syringe needle. Care was taken to inject only the tunica media with minimal perforation into the lumen of the graft. Another ($3x10^4/mm^2$) ASCs were injected into the lumen of the graft. The lumen was sealed by capping the attached barbed luers. Vascular grafts were contained in sterile T-25 flasks in a cell culture incubator in DMEM, 10% FBS, and 1% antibiotic solution. Grafts were manually turned 90° every 4 hours to ensure ASCs adequate covered the lumen surface.

4.2.4 Bioreactor Conditioning

Two vascular bioreactors were assembled, under sterile conditions, one serving as a control and the other a diabetic model. The diabetic media comprised of DMEM, 10% FBS, and 1% antibiotic solution and additional glucose was supplemented to a concentration of 5.5 g/L. Control media was prepared sans additional glucose. Static grafts were kept in T-25 flasks to serve as a control.

4.2.5 Histological Analysis

Cell viability was evaluated using Live/Dead Viability Assay Kit (Life Technologies). A small section of the graft was cut from the center. The tissue was stained according to the manufacturer procedure. The cross section was sliced and opened to expose the luminal side. Sections were imaged with fluorescence microscopy. Small sections from each graft were placed in 10% neutral buffered formalin for a minimum of 24 hours then further processed in a tissue processor. Cross sections were embedded in paraffin to examine the presence of α-SMA, SMM-hc, CD31, VE-cadherin, CCR7, and CD34 by immunohistochemistry. 5um sections were rehydrated and antigen retrieval was performed by immersing sections for 20 minutes in a 95-100°C solution of 10mM citrate buffer (pH 6.0). Next, sections were treated with 0.025% Triton X-100 solution for 10 min and blocked in 1.5% normal horse serum for 30 min. A 0.3% hydrogen solution in 0.3% normal horse serum was used to block endogenous peroxidases for 30 min. Directly following, primary antibody was applied at room temperature for one hour, followed by specific biotinylated secondary antibody (Vector Labs) for 30 min. VECTASTAIN Elite ABC Reagent, R.T.U. was applied, followed by the DAB Peroxidase Substrate Kit. For a

negative staining control, the primary antibody was omitted and segments stained only with the secondary. Sections were counterstained with hematoxylin before mounting. To visualize, digital images were obtained at various magnifications (100X to 200X) on a Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

4.3 Results

4.3.1 In-vitro Conditioning

Vascular grafts (n=5 per bioreactor) were mounted into the respective reservoir by attaching the end of the Leur fitting in the main chamber. A Cole Parmer peristaltic pump perfused media at a rate of 1 mL/min through the lumen of the graft. Culture media also filled the large reservoir to completely envelop the grafts. Grafts were maintained in the dynamic environment for up to 2 weeks in a cell culture incubator at 37°C. 0.2µm filter caps on the lids of the media reservoir allowed for sterile gas transport.

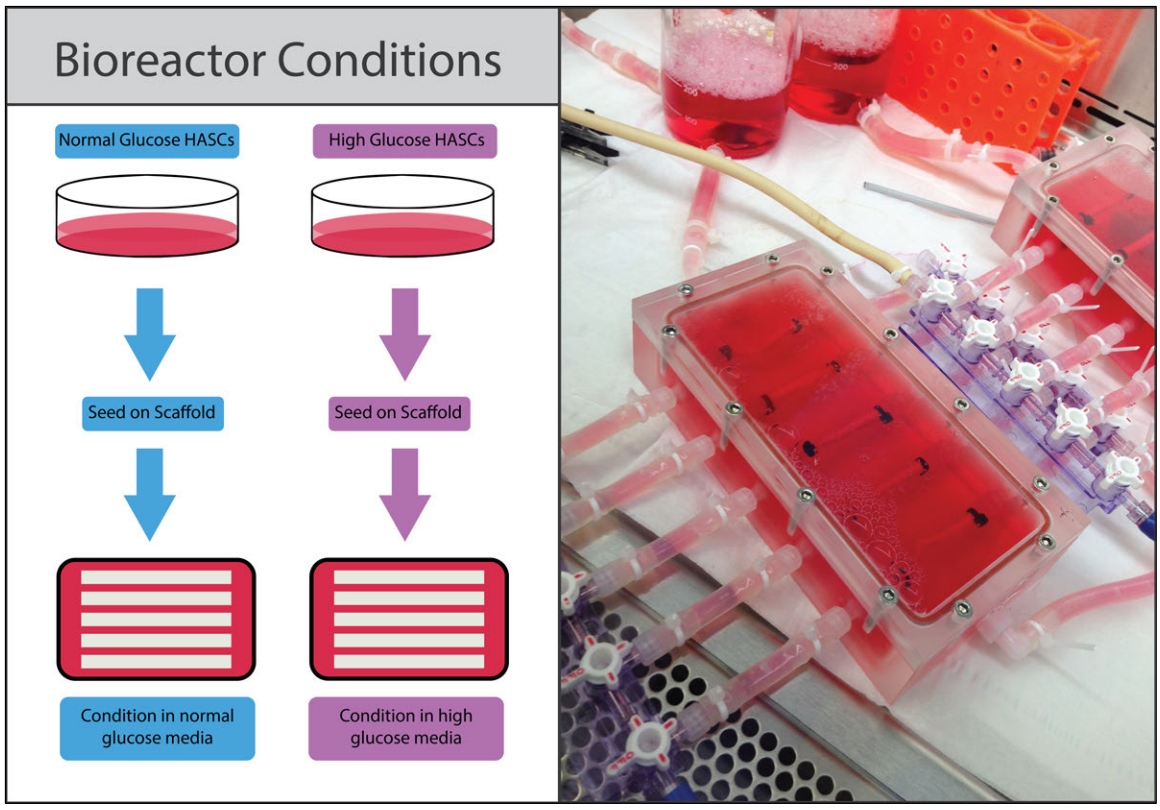

Figure 14: Vascular Bioreactor Diagram

Overview of conditions for each vascular bioreactor. Control conditions with normal media, and the other rendered diabetic condition with high glucose

4.3.2 Live/Dead Staining

Minimal live cells were observed in the static conditions as compared to the vascular grafts conditioned in the dynamic environment. Alignment of live cells in the direction of media flow was observed in both conditions. However, the alignment was more pronounced in the control conditions.

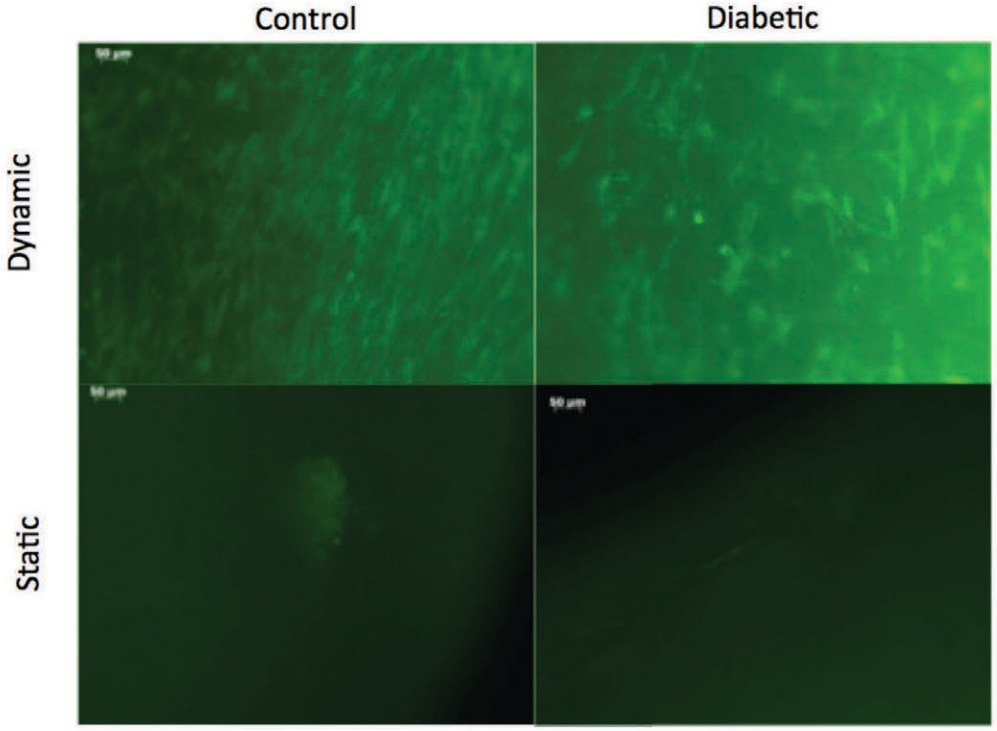

Figure 15: Live/Dead Stain of Lumen of Vascular Constructs

Calcein AM to indicate live cells and ethidium homodimer to indicate dead cells in control and diabetic dynamic conditions and statically cultured vascular grafts. Stained on lumen (*en face*). Green = Live. Red = Dead.

4.3.3 Histological Analysis

Upon examination of time zero grafts (incubation time approximately 16 hours post-seeding), hASCs were seen in boluses in the media of the grafts. Along the internal elastic lamina, a monolayer of cells was detected. When compared to the grafts after 2 weeks of conditioning, the bolus was less pronounced. The residing cells appeared to have migrated from the injection site to adjacent areas in the media. Similar results held true when examining the lumen: fewer cells remained. α-sma was noted to be present at each interval; time zero, up to 2 weeks in both the normal conditions as well as dynamic. A greater presence of α-sma was noted at time zero due to the more pronounced bolus. Elastin fibers of the matrix exhibited pseudo-positive staining for SMM-hc expression. Expression of VE-cadherin was seen at time zero in the cell bolus, as well as after 2-week dynamic culture VE Cadherin was seen along the lumen as well. Expression of CD31 by the cells remained largely absent at time zero. However, after conditioning, expression of the marker was noted on the adventitia of the graft as opposed to the lumen. The effect of the diabetic conditions is unclear. There was no noticeable shift of hASCs towards the vascular phenotypes. But it is clear that the mechanical cues and stresses the vascular bioreactor places on these grafts modulates the constructs cell retention ability and migration.

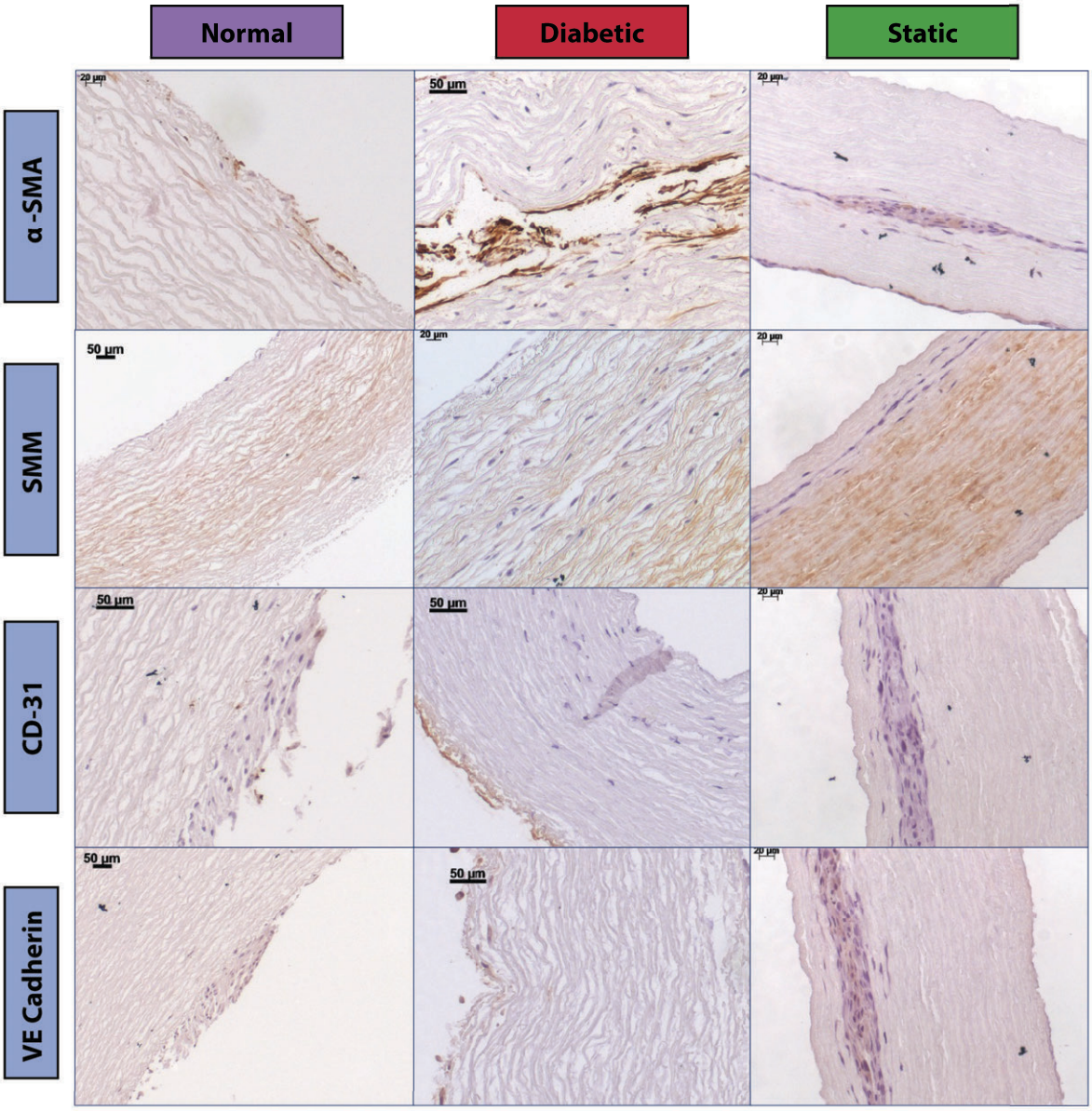

Figure 16: Immunohistochemistry of Vascular Constructs

IHC of vascular constructs at (left column) 2-week post control bioreactor conditions, (middle column) 2-week post diabetic bioreactor, and (right column) static control conditions stained for CCR7, CD31, VE-Cadherin, α-SMA, and SMM-hc. Brown = Positive. Purple = Nuclei.

4.4 Discussion/Conclusion

Our definition of diabetic in the context of this experiment is a loose term. We focus specifically on the role of hyperglycemia as seen in diabetic patients. We focused on the effects a high glucose concentration has on stem cell differentiation and viability in 3D. In this native physiological condition with relevant mechanical forces it was our aim to determine if the high glucose environment would affect the fate of our vascular grafts. Our objective was to scrutinize stem cell differentiation in 3D to determine optimum vascular graft development. The forces and stresses exhibited by bioreactors have been shown to induce differentiation of stem cells into vascular phenotypes. In our studies, we observed the presence of VE-cadherin as well as both SMC markers staining positive through the grafts. This was true despite a diabetic or normal conditions. The effects of the shear flow and mechanical stresses seem to affect the grafts in a greater manner than the diabetic conditions, potentially overshadowing any large effects due to the hyperglycemic environment. The alignment of live cells in the direction of media flow was observed in both conditions, however, the alignment was more pronounced in the control conditions.

CHAPTER FIVE: HYPERTENSIVE BIOREACTOR

5.1 Rationale

Tissue engineered blood vessels are exposed to harsh environments upon implantation in diabetic patients. Our aim is to simulate the modification of the TEVG that would occur in this pathological environment. We hypothesize that the hypertensive environment simulated in our in-vitro model will induce alterations in the structure of our TEVGs.

5.2 Materials/Methods

5.2.1 Vascular Graft Preparation

Frozen carotid arteries were obtained from Animal Technologies, Inc. (Tyler, TX), and thawed at 4°C over 24 hours. Excess fat was removed from the arteries followed by incubation in 0.1M NaOH solution at 37°C for 24 hours. This was followed by extensive rinsing with deionized water until the pH of the rinse water dropped to neutral. Scaffolds were stored in sterile 1X PBS with 1% Penicillin-Streptomycin. This treatment removed cellular epitopes and most of the collagen, leaving vascular elastin fibers unaltered.

5.2.2 In-vitro Proliferation and Differentiation of HASCs

Human adipose stem cells (hASCs) Fisher Scientific, Life Technologies) were obtained and expanded in StemPro Human AdiposeDerived Stem Cell Kit with 1% antibiotic solution (#30-004-CI, Corning – Cellgro). hASC's were cultured at sub confluent conditions on tissue culture plastic. For differentiation of hASCs to endothelial-like cells, ASCs were cultured for up to 6 weeks in EC differentiation media comprised of DMEM,

2% FBS, and 1% antibiotic solution. After sufficient cell numbers were reached, cell media was supplemented with 0.5 ng/mL vascular endothelial growth factor (VEGF, #100- 20B, PeproTech Inc) and 20 ng/mL insulin-like growth factor-1 (IGF-1, #AF-100-11, PeproTech Inc) at the time of each media change. Similarly, for differentiation of hASCs to fibroblast-like cells, cells were cultured for up to 6 weeks in fibroblast differentiation media comprised of DMEM, 5% FBS, and 1% antibiotic solution. The culture media was supplemented with 5ng/mL transforming growth factor beta-1 (TGF-β1, #100-21C, PeproTech Inc.) at the time of each media change.

5.2.3 Fibrin Gel Fabrication

Fibrinogen (#F8630, Sigma Aldrich) was reconstituted into 1X PBS and slowly dissolved at 37°C overnight before sterilization through a 0.2μm filter. Thrombin (#T7009, Sigma Aldrich) and $CaCl_2$(#BP510-100, Fisher Biotech) were combined and filtered to render sterile. Final concentration of consisted of 0.5U/mL thrombin, 4mg/mL fibrinogen, and 2mM $CaCl_2$.

5.2.4 Vascular Graft Seeding

Grafts were cut into 4-5 cm sections and fittings were chosen to closely match inner diameter to the lumen of the artery. O-rings were used to securely attach the graft to the fitting. The pre-differentiated endothelial cells were re-suspended in the thrombin calcium mixture. Immediately before injection, the thrombin mixture was combined with the fibrinogen to initiate the formation of the fibrin gel. Silicone plugs were used to cap one side of the graft and a total of ($2.6x10^4/mm^2$) pre-differentiated endthoelial HASCs

were injected into the lumen of the artery. The remaining end of the artery was plugged with a silicone plug to prevent leakage of the cell mixture. This was repeated for each subsequent graft for each bioreactor, including a graft to be kept as a static control. Each vessel was placed in a sterile 50mL conical with culture medium to prevent the grafts from drying out. Each conical was then securely attached to a rotating device and set to rotate 0.5 revolution per minute for 1 second and to hold in between rotations for 15 seconds. This procedure continued over 2 hours to ensure adequate cell attachment and even fibrin gel distribution across the lumen. Following this, ($1x10^4/mm^2$) pre-differentiated fibroblast HASCs were drop seeded onto the adventitia of each graft. To minimize cell loss, silicone tube acted as channels for the

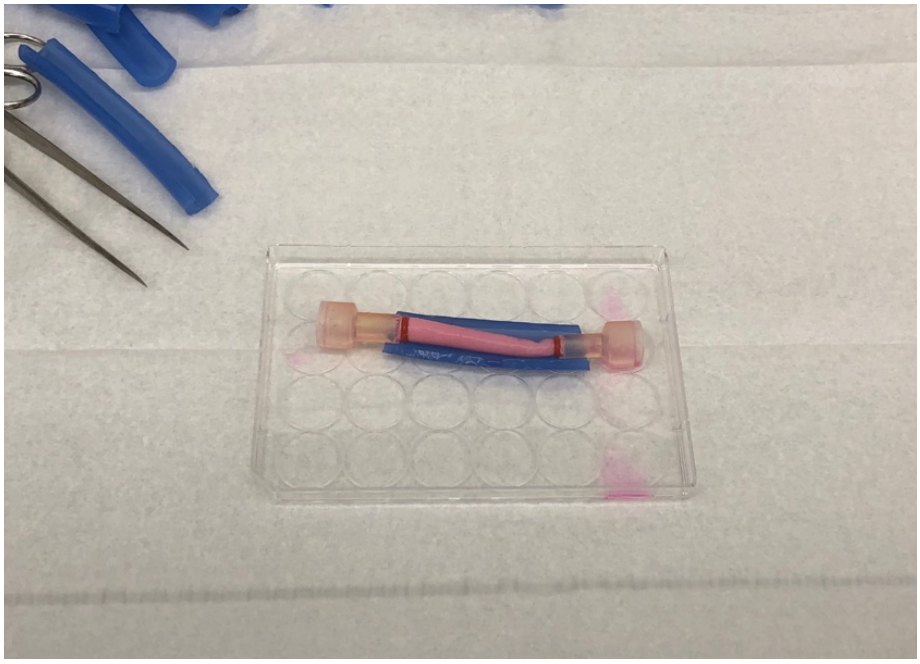

Figure 17: Seeding of Vascular Graft

Silicone tubing used as a trough during drop seeding of pre-differentiated Fibroblast HASCs onto the vascular graft.

grafts to rest in and catch any run-off. Cells were allowed to attach for 1 hour before rotation was resumed and additional culture medium added to the grafts in the conical.

5.2.5 Bioreactor Conditioning

Following seeding, each bioreactor was assembled in a sterile hood. The primary method of sterilization was steam sterilization save the acrylic chamber and hand tools that required sterilization by ethylene oxide gas. Culture medium composed of DMEM, 10% FBS, and 1% antibiotic solution was used to fill each bioreactor. 5 vascular grafts were mounted into each bioreactor and sealed. The system was placed in a cell culture incubator to maintain appropriate temperatures and gas levels. Additional Penicillin-Streptomycin was added every 3-4 days and the culture medium replaced every 7 days. Grafts were housed for 4 weeks before removal for analyses.

5.2.6 Mechanical Testing

MTS Synergie 100 Single Column Universal Test Machine was used to determine the radial elastic modulus of the conditioned vascular grafts. 1cm sections from the bioreactor-conditioned vascular grafts were cut opened as depicted in the **Fig.17** and flattened to yield a 1 cm x 3 cm rectangular sample shape. The loading blocks of the machine were lined with sandpaper and a portion of each end of the artery was clamped to prevent slippage. Final elastic modulus was determined for n=4 samples.

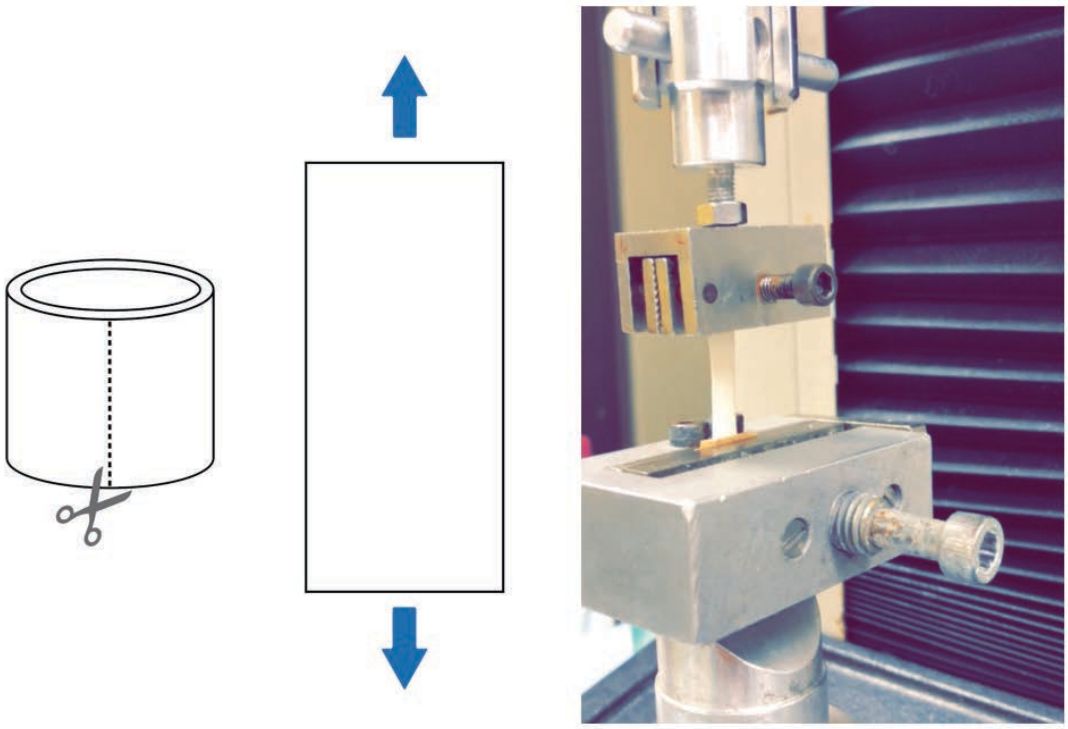

Figure 18: Mechanical Testing of Bioreactor-Conditioned Vascular Graft

Samples of the bioreactor-conditioned vascular grafts are cut and oriented as shown in the diagram to the left. Top and bottom ends are secured with sandpaper in the loading blocks as force is applied.

5.2.7 Histological Analysis

Cell viability was evaluated using Live/Dead Viability Assay Kit (Life Technologies). A small section of the graft was cut from the mid-section of the graft. The tissue was stained according to the manufacturer procedure. The cross section was sliced and opened to expose the luminal side. Sections were imaged with fluorescence microscopy. Small sections from each graft were placed in 10% neutral buffered formalin for a minimum of 24 hours then further processed in a tissue processor. Processed tissues were embedded in paraffin and at 5um sections were stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome according to manufacturer protocols (Poly Scientific R&D Corp) for a general overview of morphology.

5.3 Results

5.3.1 In-vitro Bioreactor Conditioning

Following seeding, each bioreactor was assembled in a sterile hood. The main chamber was filled with culture medium before attaching each graft. Grafts were secured to the stainless steel through-wall fittings with the protection of an O-ring securing each fitting. Following the securement of the final graft, the main chamber was sealed with the lid and an o-ring and bolted into place. Culture medium was slowly added to fill the entire bioreactor before sealing the entire flow loop. Each bioreactor was attached to a separate Aptus vascular system. The system served to establish separate pressures for each condition, to control the pump and monitor. Care was taken to ensure cell attachment by slowly ramping up the conditions of the bioreactor as seen in **Fig.18**. The internal pump was set to a flow rate of 3% power of the pump which translates to 10 ml/min. Over the

course of a week the intervals of the pinch valve were slowly decreased to a final pace of 500ms open, 300ms closed. Upon ramping up the pressure in the second week, the flow of the pump was increased to 5%, or 18 ml/min in order to achieve the desired pressures. Over the course of a week pressures in the normal and hypertensive bioreactor were ramped to a final pressure of 120/85 and 140/90 respectively. The goal was to run the bioreactor for approximately 2 weeks at the final physiologically relevant pressures to provide enough exposure time at the maximum pressures.

Bioreactor Schedule			
Date	Action	Pressure Reading [mmHg]	
11.14.17	Flow set to 3%	Normal	Hypertensive
11.18.17	Pinch Valve 1000/600	7.8/2	9.1/1.2
11.20.17	Pinch Valve 900/500		
11.21.17	Pinch Valve 800/500		
11.22.17	Pinch Valve 700/400		
11.24.17	Pinch valve 500/300	14/6	21/8
11.27.17	Flow set to 5%	32/10	32/26
11.28.17	Pressure Modulated	55/30	75/20
11.30.17	Pressure Modulated	80/45	100/50
12.1.17	Pressure Modulated	120/85	140/90
12.13.17	End of Bioreactor		

Figure 19: Bioreactor Conditioning Regime for Ramp-Up

To ensure adequate time for cell to adjust to the pressures and flow, care was taken to slowly ramp up conditions in the bioreactor.

Each bioreactor, as shown in **Fig.17**, connected to a separate computer controlled system for greater control over flow and pressures to apply correct pressures and flow for each condition. As shown, this new compact system with internal peristaltic pump easily fits two separate setups into a standard cell culture hood. Each system was controlled by a separate computer running Aptus Vascular Bioreactor Software. Each system possessed the capability to simultaneously monitor 2 pressure transducers, one flow meter and could be programmed to modulated a pinch valve and a peristaltic pump.

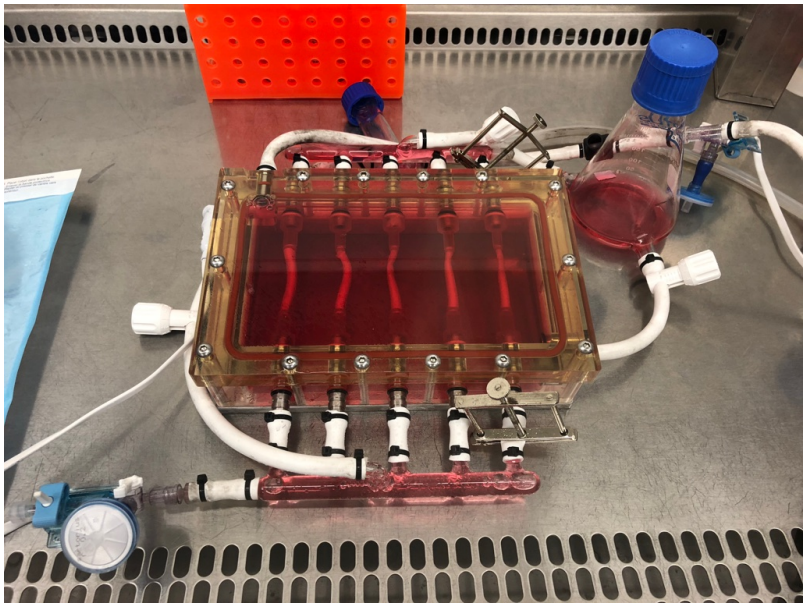

Figure 20: Mounting Seeded Vascular Graft into Bioreactor

Five vascular grafts mounted into assembled bioreactor and sealed. Culture medium fills chamber and reservoir to bathe the grafts.

Figure 21: Cell Culture Incubator Housing Two Bioreactors

Two complete Aptus bioreactor systems easily fit enclosed in a cell culture incubator. Controlling units are connected by feeding wires through the incubator.

Aptus Vascular Software was used to control the peristaltic pump and the pinch valve. Pump speed was set as a percentage of the voltage of the pump which results in a linear increase in speed of the roller heads of the pump. The flow in the bioreactor was monitored with a flow meter and could be displayed on the software. The two transducers provided a reading to output as shown in **Fig.21**. Displays of the pressure profiles after completion of the ramp up cycle are shown of both the normal and hypertensive conditions.

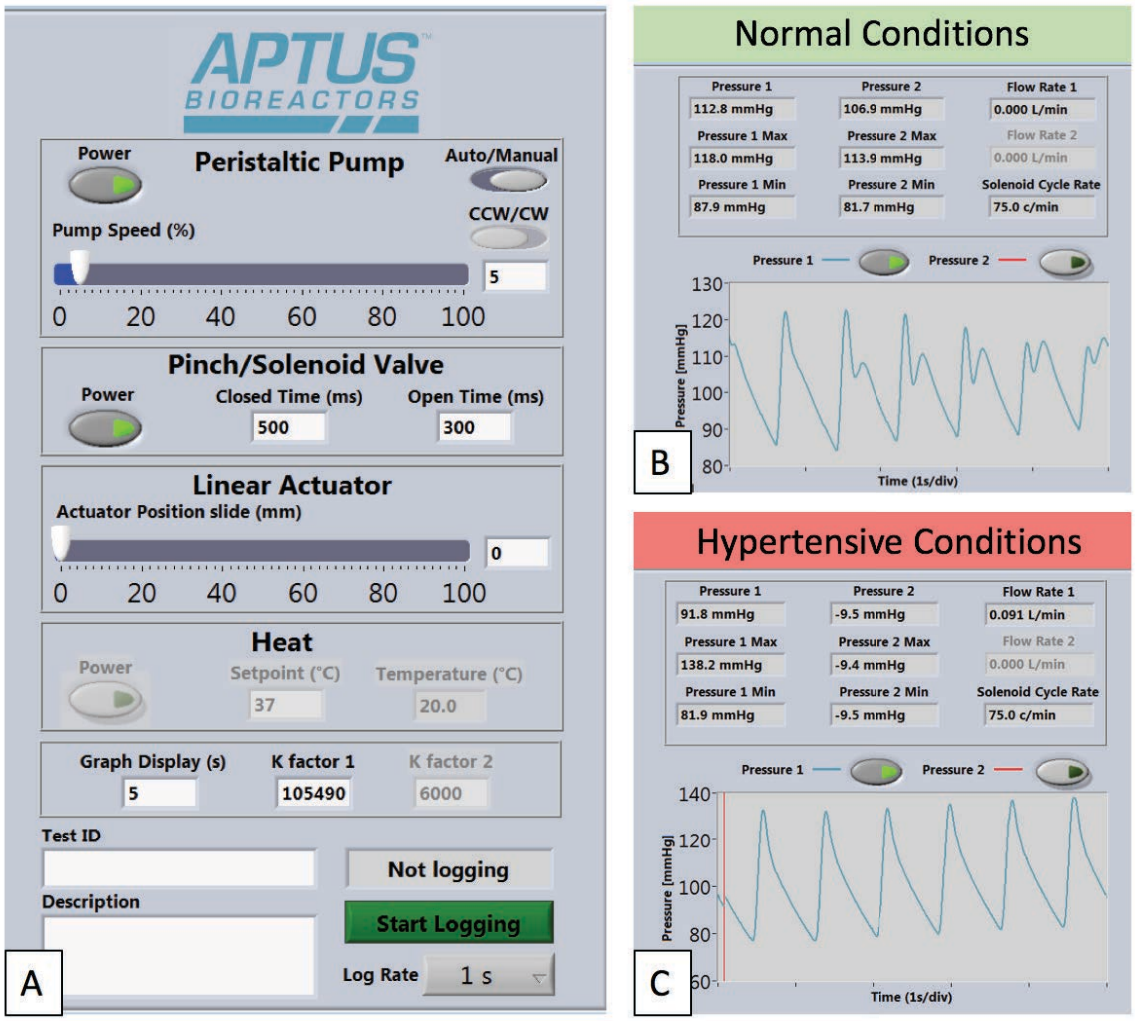

Figure 22: Pressure Profile of Bioreactors

Snapshot of Aptus Vascular Bioreactor Software shows main controlling display (A) to set peristaltic pump speed and pinch valve delay. Display of the normal conditions (B) and hypertensive conditions (C) is shown at the end of the ramp up cycle.

5.3.2 Differentiation of HASCs to Vascular Phenotypes

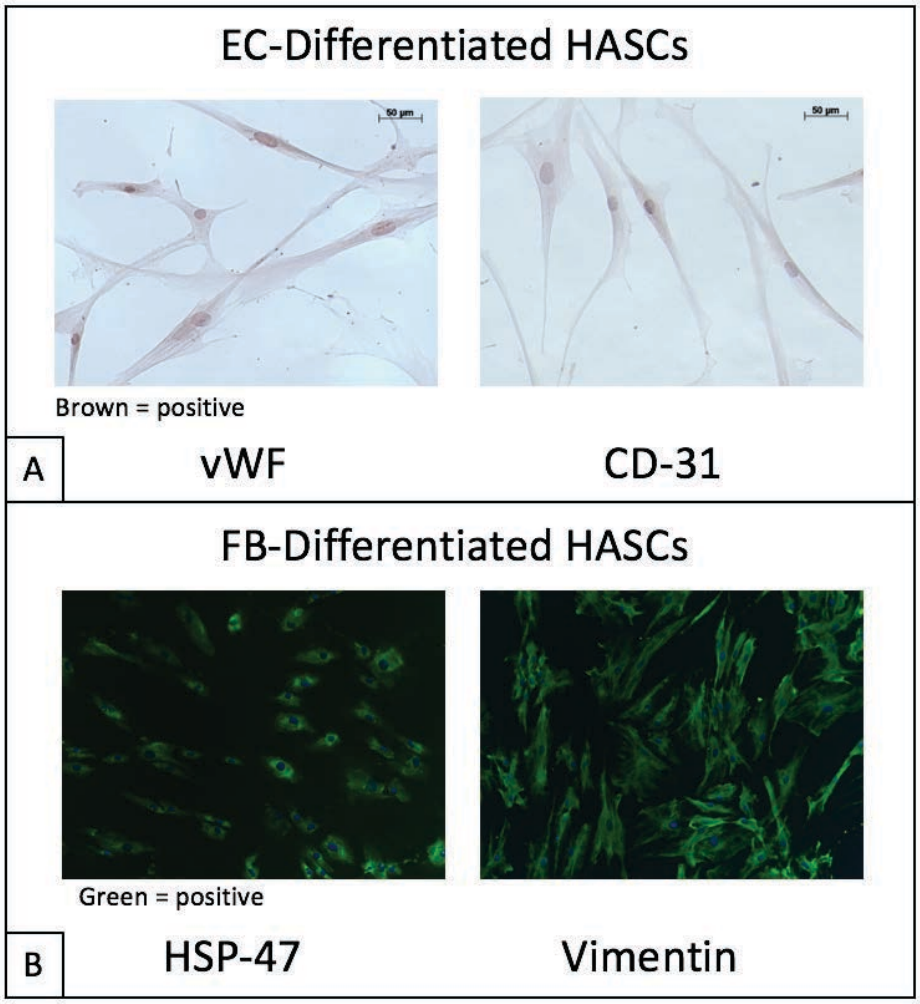

Figure 23: Immunocytochemistry and Immunofluorescence of Pre-Differentiated HASCs

Immunocytochemistry (A) results shown for endothelial-cell (EC) differentiated HASCs. CD-31 and Von Willebrands's Factor (vWF) were stained for. (brown = positive. Immunofluorescence (B) performed on fibroblast (FB) differentiated HASCs. Cells were stained for markers (HSP-47) and Vimentin. (green = positive, images taken at 10X magnification)

HASCs were pre-differentiated in 2D culture prior to seeding on prepared vascular grafts. Immunocytochemistry and immunofluorescence was performed to investigate the potential of HASCs to differentiate to vascular phenotypes. The results of the staining is shown in Fig.22

5.3.3 Live/Dead Staining

Live/Dead was performed on seeded vascular grafts kept at static conditions during the operation of the bioreactors. Cells were still present in both the lumen as well as the adventitia, however cells death was also seen in both locations as well. Some spreading of the cells was noticed in the adventitia however minimal was noticed with the EC differentiated cells.

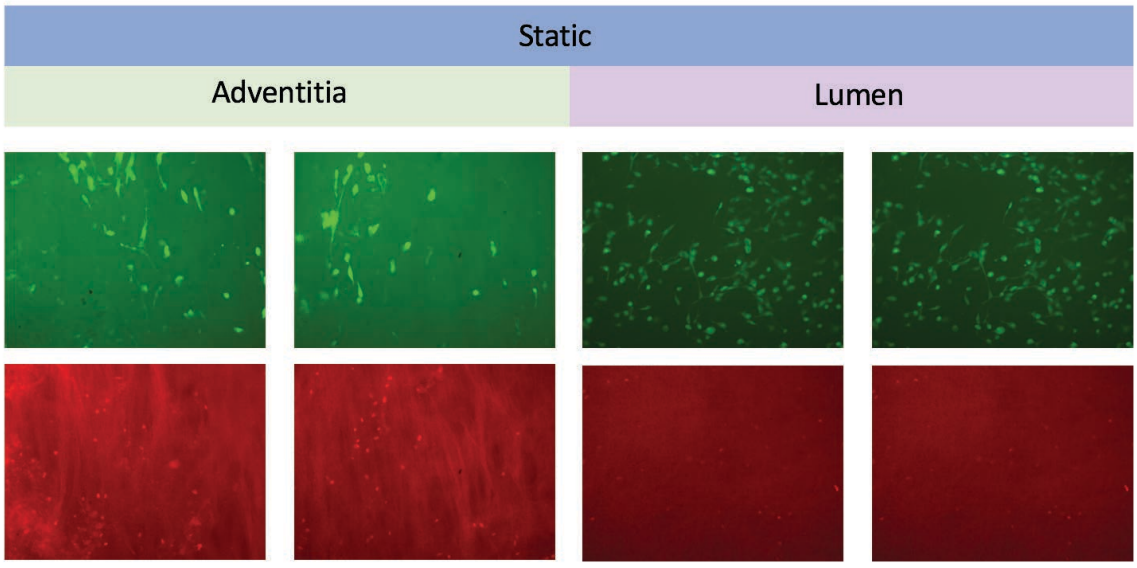

Figure 24: Live/Dead Evaluation of Static Vascular Graft

Live/Dead images of static vascular grafts. Grafts were cut and lain flat to obtain images from the luminal side and the adventitial side.

Live/Dead staining of the vascular grafts following removal from the bioreactor confirmed presence of cells in both the normal and hypertensive vessels. Cells were found in both the lumen and adventitia, however cell presence was lessened as compared to time zero and static conditions. The cells in both conditions and in both the adventitia and lumen appeared to be elongated in the direction of the flow.

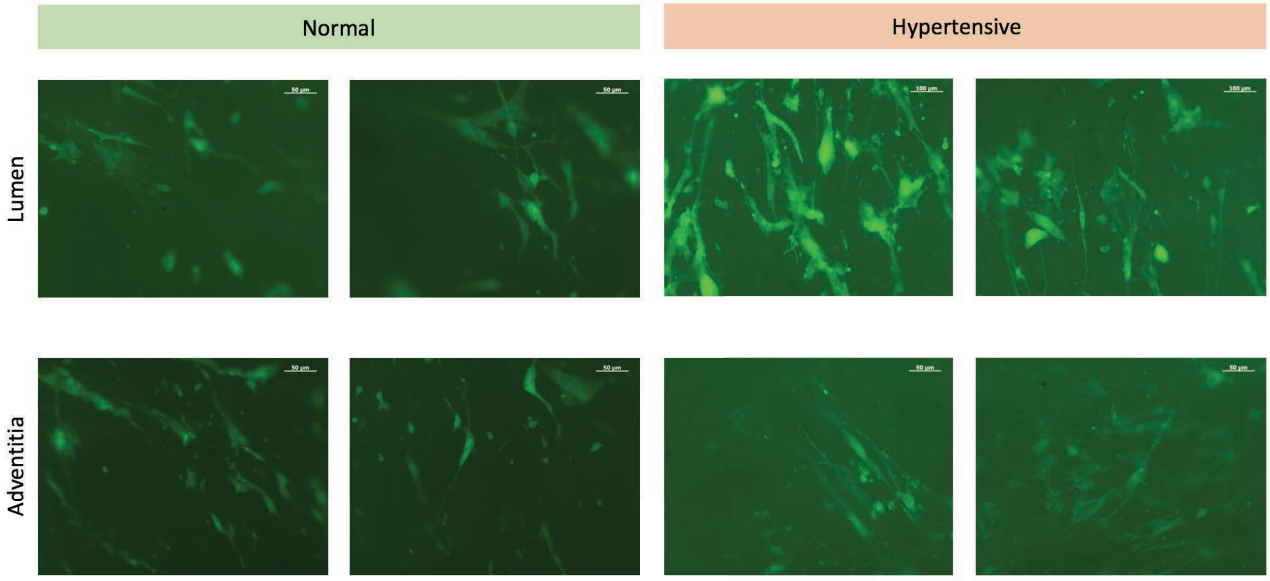

Figure 25: Live/Dead Evaluation of Bioreactor-Conditioned Vascular Grafts

Live/Dead images of bioreactor-conditioned vascular grafts. Grafts were cut and lain flat to obtain images from the luminal side (top row) and the adventitia (bottom row) from both the normal group and hypertensive bioreactor conditions.

5.3.4 Histological Analyses

H&E staining was performed on the of the grafts following bioreactor conditioning as shown in **Fig.24**. Some cells could be seen lining the endothelium, but more cells may be present on further sections. Images revealed general maintenance of integrity of the tissue. However, in the hypertensive group, the outer lamellae fibers showed signs of fraying. Masson's Trichrome staining was performed to investigate the relative levels of collagen and elastin. The images from the hypertensive group visually appeared to possess a greater presence of collagen as shown in **Fig.25** as compared to the normal group.

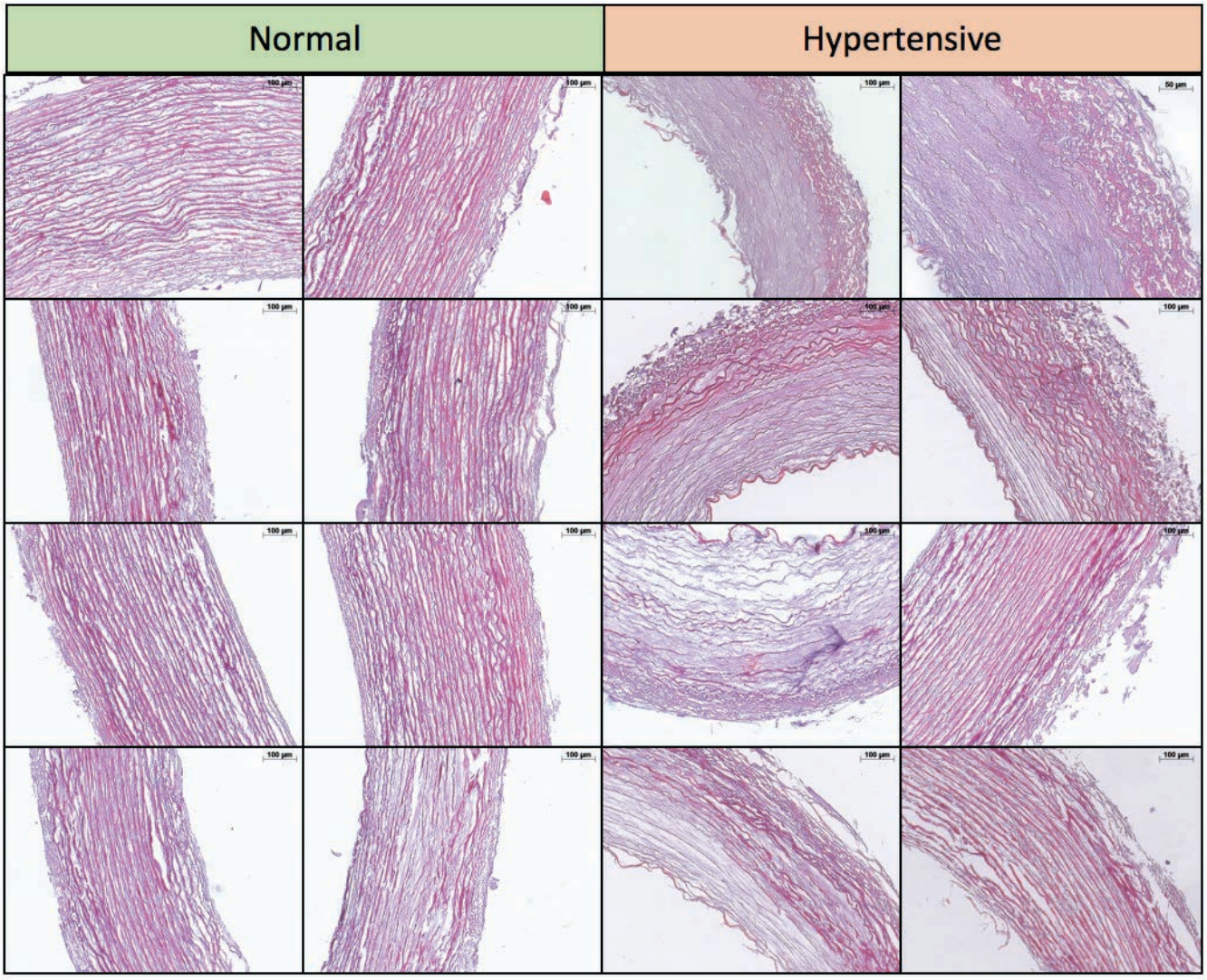

Figure 26: Hematoxylin and Eosin staining of Bioreactor-Conditioned Vascular Grafts

H&E staining of both the normal (left two columns) and hypertensive bioreactor-conditioned grafts (right two columns). (pink=ECM purple = nuclei)

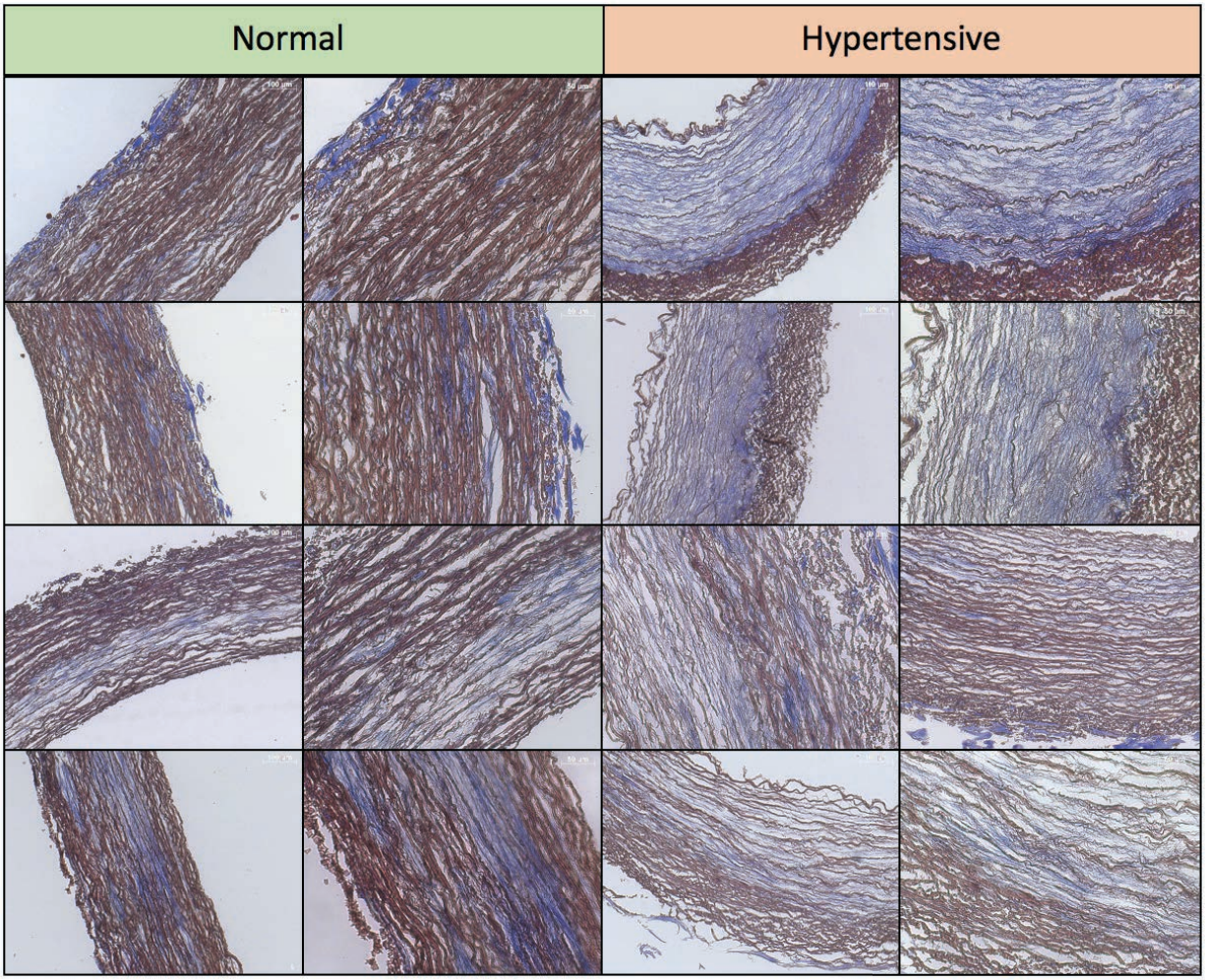

Figure 27: Masson's Trichrome Staining of Bioreactor-Conditioned Vascular Grafts

Masson's trichrome staining of both the normal (left two columns) and hypertensive bioreactor-conditioned grafts (right two columns). (brown=elastin, blue = collagen)

5.3.5 Collagen Quantification

The images captured from the Masson's staining were used to quantify the percentage of collagen content. Images were processed using ImageJ and the results as seen in **Fig.26** show a significant increase between the normal and hypertensive bioreactor group.

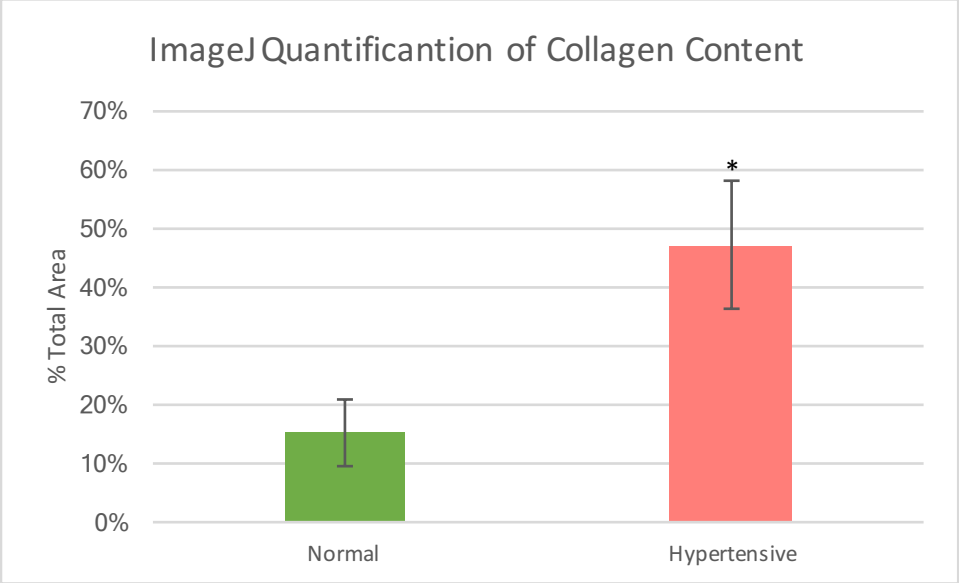

Figure 28: Collagen Content Quantification of Bioreactor-Conditioned Vascular Grafts

Collagen content was determined by quantifying the percentage of total area of collagen to total extracellular matrix composition. Images were quantified in ImageJ by color threshold determination. (n=7)
**indicates statistical significance*

5.3.6 MTS Quantification

After 4 weeks conditioning, grafts underwent unidirectional mechanical testing to determine the elastic modulus of the conditioned grafts. As seen in **Fig.27,** the hypertensive grafts had a significantly higher modulus as compared to the grafts in the normal group.

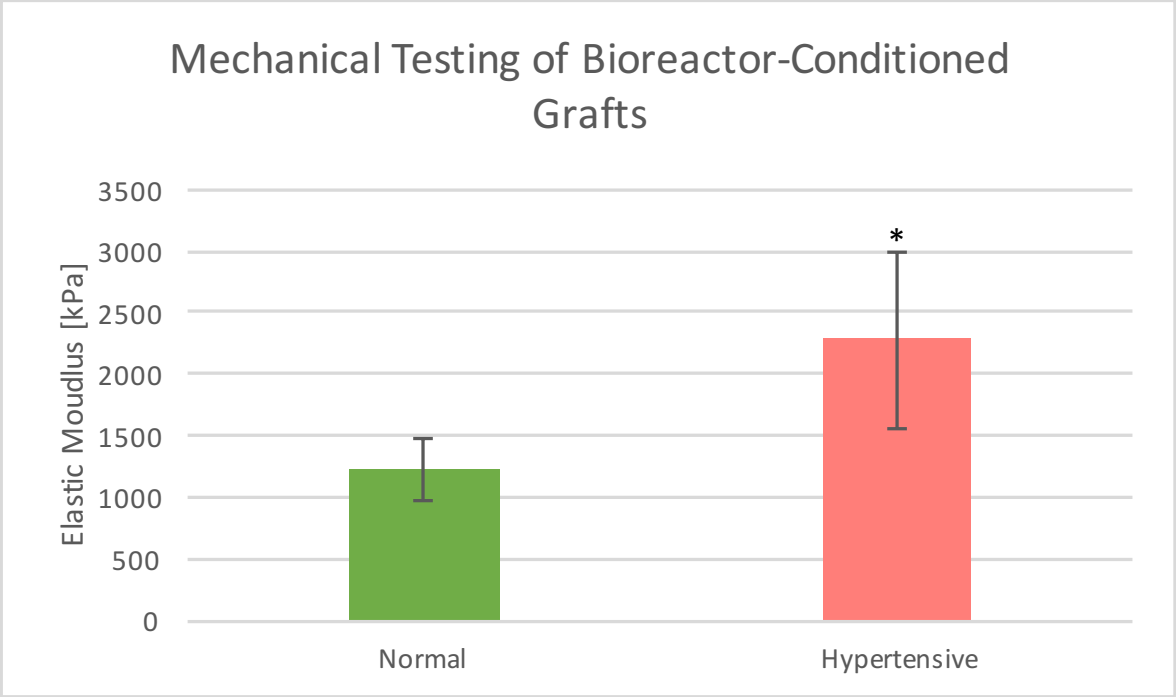

Figure 29: Elastic Moduli of Bioreactor-Conditioned Vascular Grafts

Elastic modulus was determined for each group, normal and hypertensive grafts. (n=4)
**indicates statistical significance*

5.4 Discussion/Conclusion

Our development of the newly outfitted bioreactor performed well with new modifications, was easily sterilized, and its compact nature was beneficial as two simultaneous systems needed to be housed in one cell culture unit. The new Aptus Bioreactors units were user-friendly, easy to assemble and greatly simplified the task of monitoring the data from multiple sensors. During conditioning, the distal portion of the grafts, which were thinner by nature were prone to slits forming from the pressures. It is possible this is due to the pressures alone, however it is possible there was some damage done to the grafts when mounting that could have exacerbated the tearing. Because of this the profile of the pressures had to be modulated in as a best a way to maintain appropriate systolic and diastolic pressures. The wave forms suffered from the tears in the arteries, but pressures were able to be maintained.

Live/Dead analyses of the grafts after conditioning showed cell retention in both the adventitial and luminal face. When compared to the static control, a seeded graft not subjected to dynamic conditioning, there was less cell retention. The static controls showed dead cells on the grafts, however with the dynamic conditioned grafts, dead cells were not located as they were likely removed by the shear of the flow after detaching. It was noted that the dynamic grafts showed spreading of cells, specifically on the luminal side, whereas the static samples had random orientation and no specified spreading or alignment. This finding agrees with the previous bioreactor as well as many groups using similar systems. [47]

It was noticed in our studies that the subjection of our TEVGs to the hypertensive environment induced a pathological stiffening. This is likely due to the over presence of collagen in the extracellular matrix. Indeed, this result was qualitatively visualized with Masson's staining of sections. Collagen seemed to increase significantly in the hypertensive group and this finding agreed with ImageJ quantification of the images. Color thresholding separation showed a significant increase in hypertensive samples with collagen comprising approximately half the extracellular matrix of the grafts. The detection method used with ImageJ can be used to detect the dominant color, however if tissues lie on top of each other, quantification may not be as precise. Further analysis may be needed for conclusive evidence. However, this response of increased collagen production is not shocking, as it has been shown that dynamic conditioning of vascular grafts leads to an increased collagen production and fiber density. ECM is produced by the cells of the vascular matrix and indeed, studies have shown that increased circumferential stretch leads to an increase of extracellular matrix production [48]. As well, the elastic modulus of the grafts in the hypertensive group was shown to be significantly higher than the normal pressures group. Although less cells remained than initially seeded, cells were still found to be present on both the adventitia and lumen of the grafts. Cells were conditioned on the graft for 2 weeks at lower conditions before a slow ramp up, providing time for deposition of collagen by the attached cells. It was also noticed that the lamellae of the elastin fiber of the hypertensive groups seemed frayed and degraded. This has been shown to be a result of elevated levels of pressure and flow.

Based on these results, we believe the systematic increase of the pressure within the bioreactor is providing mechanical cues to the vascular graft resulting in alterations to the matrix as expected in a similar physiological environment.

CHAPTER SIX: FINAL OVERALL CONCLUSIONS

6.1 Conclusions

We have developed an in-vitro vascular graft bioreactor to be used in the conditioning and testing of tissue-engineered vascular grafts. Additions to the system now allow a variety of parameters to better model the pathological environment these grafts would eventually be subjected to in-vivo. We have shown our bioreactor can elicit a typical response of the vascular grafts as seen in a hyperglycemic and hypertensive environment. These results are promising as the use of the in-vitro model can reduce the burden of animal models through testing in a simulated environment.

6.2 Future Directions

In these experiments we focused on the hyperglycemic and hypertensive conditions separately with each iteration of the vascular bioreactor. However, hyperinsulinemia and hyperlipidemia are metabolic conditions associated with diabetes. For future in vitro studies, a combinatory effect should be investigated. Insulin and free fatty acids should be added to the media to for a more representative view of the effect of biochemical cues on the TEVG. As well, this should be investigated in combination with the increased pressures to better model the mechanical effects a hypertensive environment has on the grafts. Finally, for further in vivo characterization of the effect diabetes has on our tissue engineered vascular grafts, use of a diabetic animal model for a more complete picture of how the grafts are affected would be necessary.

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